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A Study of the Carotenoids Echinenone and Myxoxanthin with Special Reference to their Probable Identity

By T. W. GOODWIN and M. M. TAHA
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(*Received* 4 *October* 1950)

It has recently been shown (Goodwin & Taha, 1950) that the ketocarotenoid present in the gonads of the limpets *Patella vulgata* and *P. depressa* is identical with the pigments isolated from the gonads of the sea-urchin *Paracentrotus lividus*, and from the blue fresh-water alga *Aphanizomenon flos-aquae*, and termed respectively echinenone (Lederer, 1935) and aphanin (Tischer, 1938, 1939). (In this paper the term echinenone will be used.) It was also suggested that echinenone was probably identical with the carotenoid, myxoxanthin, characteristic of the blue-green algae (Myxophyceae), which was isolated crystalline from the fresh-water alga *Oscillatoria rubescens* (Heilbron & Lythgoe, 1936; Karrer & Rutschmann, 1944). This identity had previously been suggested by Lederer, but considered improbable by Heilbron (see Karrer & Jucker, 1948, p. 335). It has recently been possible to obtain a small amount of myxoxanthin from a mixed culture of *Oscillatoria* spp. and to compare it directly with a sample of echinenone.

EXPERIMENTAL

Extraction of pigments. The sample of mixed *Oscillatoria* spp. was covered with 20% (w/v) ethanolic KOH and allowed to stand for 24 hr. at room temperature, under N_2 and in the dark. The mixture was then filtered through a Büchner funnel and the green filtrate transferred to a separating funnel. After addition of an equal volume of water the carotenoids were removed by extraction with four successive portions of ethyl ether freshly distilled over reduced Fe. The combined ether extracts were washed free from alkali, dried over anhydrous Na_2SO_4 and the ether removed *in vacuo*; the residue was dissolved in a small volume of light petroleum (b.p. 40–60°).

Separation and purification of myxoxanthin. The light petroleum solution was extracted three to four times with an equal volume of 90% (v/v) aqueous methanol and the methanol fractions (the hypophasic pigments) discarded. The petroleum fraction was taken to dryness *in vacuo*, the residue dissolved in a little methanol, and cooled in solid CO_2. The steroids which crystallized out were removed by filtration through a Büchner funnel packed with solid CO_2. The methanol was then removed *in vacuo* at about 30° and the purified residue dissolved in light petroleum (b.p. 40–60°) and chromatographed on alumina (Spence, Grade O) deactivated with methanol (Goodwin & Taha, 1950). Two zones were obtained, the upper being brick-red and the lower orange and diffuse; the latter was eluted with light petroleum (b.p. 40–60°) containing 1–2% (v/v) of ethyl ether, and shown to be β-carotene. The brick-red zone was eluted with ethyl ether and the solvent removed; the residue was dissolved in light petroleum and the fraction rechromatographed. The procedure was repeated twice and the purified product compared chromatographically and spectroscopically with a similarly purified specimen of echinenone prepared from *Patella* gonads.

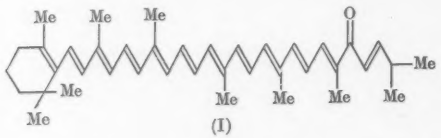
Derivates of myxoxanthin. Two derivates of myxoxanthin, the oxime and the alcohol, prepared by reduction with aluminium *iso*propoxide, were prepared in a chromatographically homogeneous state as described by Goodwin & Taha (1950) for echinenone. They were also compared chromatographically and spectroscopically with the corresponding derivates of echinenone. The reduced product was also compared with a chromatographically pure specimen of cryptoxanthin obtained from yellow maize.

RESULTS AND DISCUSSION

Spectrographically, the sample of myxoxanthin obtained in the present investigation was indistinguishable from echinenone; similarly, the corresponding derivatives, the oxime and the alcohol, were also indistinguishable from those of echinenone; the positions of the absorption bands were identical with those previously recorded for echinenone and its derivatives (Goodwin & Taha, 1950) and are not recorded here. For illustration, however, the complete visual absorption curves for myxoxanthin and echinenone are recorded in Fig. 1; it will be seen that they are practically identical over the whole of this spectral range. When compared with echinenone using the mixed chromatogram technique (Goodwin & Taha, 1950), no separation of the two pigments could be obtained; furthermore, no chromatographic separation of mixtures of the oximes or the alcohols could be demonstrated. Considering this evidence, there is, therefore, no reasonable doubt that echinenone and myxoxanthin are identical.

Goodwin & Taha (1950) have shown that spectroscopic evidence indicates 4-keto-β-carotene as the probable structure of echinenone, and at the same time pointed out that, even if myxoxanthin and echinenone were not the same pigment, the structure (I) assigned to myxoxanthin (Heilbron & Lythgoe, 1936; Karrer & Rutschmann, 1944) was not tenable on the spectroscopic evidence; the present results indicate that this decision was justified. One additional point concerning struc-

ture (I) should perhaps be made; this structure was finally accepted on an interpretation of spectral data which cannot now be considered valid. It was assumed that the single-banded



(I)

spectrum of this and other ketocarotenoids is due to the simultaneous conjugation of the carbonyl group with two sets of unsaturated linkages (Heilbron & Lythgoe, 1936). It is now known that this

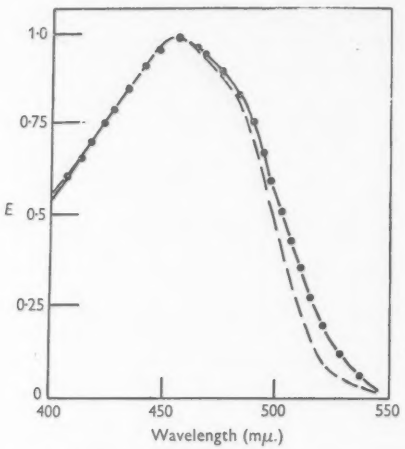


Fig. 1. The absorption spectra of echinenone (----) and myxoxanthin (-·-·-) in light petroleum (b.p. 40–60°). Scales for the extinctions (E) have been so chosen that the ordinates representing $E_{max.}$ for the two pigments are equal (1·0).

is not a necessary condition for the production of a single-banded spectrum, for such a spectrum is exhibited by astaxanthin (Goodwin & Srisukh, 1949) which is 3:3′-dihydroxy-4:4′-diketo-β-carotene (Kuhn & Sörensen, 1938).

It has been shown in the present work that the Oppenauer-reduction product of echinenone is chromatically indistinguishable from cryptoxanthin. Previously (Goodwin & Taha, 1950), they had only been compared spectroscopically. This provides further evidence in favour of considering cryptoxanthin to be 4-hydroxy- and not 3-hydroxy-β-carotene. There is no compelling reason for accepting 3-hydroxy-β-carotene as the structure, for it is based on an analogy which is not too secure (see Karrer & Jucker, 1948, p. 178). It is only the 3′-hydroxyl group, in the α-ionone ring, which is unequivocally fixed in lutein (generally accepted as 3:3′-dihydroxy-α-carotene; see Karrer & Jucker, 1948, p. 205), the other position (3) in the β-ionone ring, is fixed by analogy; the 3-position in cryptoxanthin is then fixed by analogy with the doubtful position of the hydroxyl group in the β-ionone residue of lutein. This 'analogy of symmetry' cannot be considered to carry a great deal of weight because the lutein molecule is asymmetrical. It is at least as logical to argue that the plant, in synthesizing lutein, would maintain the same relative positions of the double bond and the hydroxyl group in both the α- and β-ionone residues. On this reasoning lutein would then be 3′:4-dihydroxy-α-carotene and cryptoxanthin 4-hydroxy-β-carotene. There is thus good reason to believe that 4-hydroxy-β-carotene best represents the structure of cryptoxanthin, but final proof of this depends on extensive degradation studies.

SUMMARY

1. The carotenoid myxoxanthin, its oxime and its Oppenauer-reduction product (alcohol) are spectroscopically and chromatographically indistinguishable from echinenone and its corresponding derivatives; it is therefore concluded that the pigments are identical.

2. The Oppenauer-reduction product of echinenone is spectrographically and chromatographically identical with cryptoxanthin. This strongly suggests that the structure of cryptoxanthin is 4-hydroxy- and not 3-hydroxy-β-carotene.

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Relationship between the Structure of the Simple Sugars and their Behaviour on the Paper Chromatogram

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(*Received* 28 *July* 1950)

The object of the present study was to relate the chemical structure of the sugars to their R_F values on the paper chromatogram.

In the case of a homologous series the relationship between the R_F value and the structure is particularly simple. Consden, Gordon & Martin (1944), in their work on the separation of the amino-acids, have shown that the R_F values of members of a homologous series change in a regular manner with each additional group in the molecule. Martin (1949), treating the paper chromatogram as a liquid/liquid extraction column, has predicted on theoretical grounds that a linear relationship should exist between the logarithm of the partition coefficient of a substance between the two phases on the paper and the number of active groups of any one kind in the molecule, provided that the various active groups do not interfere with each other. The partition coefficient is calculated from the relation (Consden *et al.* 1944)

$$\alpha = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right), \qquad (1)$$

in which

$$R_F = \frac{\text{movement of band of substance under investigation}}{\text{movement of the advancing front of liquid}},$$

A_L = cross-sectional area of solvent phase,

A_S = cross-sectional area of water phase,

$$\alpha = \text{partition coefficient}: \frac{\text{concentration of solute in aqueous phase}}{\text{concentration of solute in the organic phase}}.$$

Since A_L/A_S is assumed to be constant for a given solvent and paper at a given temperature, α is directly proportional to $([1/R_F]-1)$, and the relationship deduced by Martin (1949) mentioned above can be rewritten with $\log([1/R_F]-1)$ in place of $\log \alpha$. Bate-Smith & Westall (1950) have tested this relationship for a number of homologous series of substituted aromatic compounds and have found good agreement. It is probable, however, that the picture of the paper chromatogram as a liquid/liquid extraction column is over-simplified, because the water imbibed into the cellulose fibres is almost certainly bound to the cellulose and is not free in the sense required by Martin's theory. In addition, it is probable that, in certain cases at least, the free hydroxyl groups of the cellulose are active. A more comprehensive picture, especially for hydrophilic solutes, has been suggested by Hanes & Isherwood (1949). These authors regard the operation of the chromatogram as depending upon the relative ease with which the solutes can enter into the structure of the water imbibed on the cellulose and into the structure of the water in the mobile phase. Such a picture gives a more concrete expression to the influence of the molecular size, shape and disposition of active groups in the solute on the R_F value. The partition coefficient α in equation (1) is then regarded as a measure of the distribution of a solute between the mobile phase and the water-cellulose complex.

The purpose of the present study is to relate the chemical structure of the sugars to their movement on the chromatogram. An important part of the study is the elucidation of the influence of the configuration of the hydroxyls of the sugars, for a preliminary survey of the experimental data shows that the separation of the simple sugars must depend to a large extent upon the spatial arrangements of the hydroxyl groups and the interaction between them, and only to a small extent upon the number of hydroxyl groups present. This follows from the fact that whole groups of sugars, which are separable on the chromatogram, have the same number of hydroxyls and probably even the same ring structure. Study of the sugars offers, therefore, the possibility of exploring the effect on the R_F value of different arrangements of the same groups in the molecule. However, before a more detailed analysis of the experimental data can be made, it is essential to obtain some idea of the probable structure of the sugars in solution. In the case of some of the common pentoses and hexoses which exist in the crystalline state as pyranoses, Cox, Goodwin & Wagstaff (1935)

suggest that there is little change in ring form during the process of solution. They point out that there is a close correspondence between the molecular volume in the solid state and the aqueous solution volume, which indicates that the form of the molecule is constant. This suggestion receives some support from work on the oxidation of the aldoses to the corresponding δ-lactones (Pigman & Goepp, 1948) which indicates that the common hexoses have pyranose ring structures in solution. Additional evidence that a number of sugars are almost entirely in the pyranose form in solution has been provided by a study of the mutarotation of sugars in aqueous solution. Isbell & Pigman (1937) have shown that for a number of aldohexoses the reaction follows a first-order equation, and that this fact makes it probable that the main constituents of the equilibrium solution are the α- and β-pyranose modifications. However, a number of sugars exhibit mutarotations which do not follow the first-order equation, and it is suggested by Isbell & Pigman (1937, 1938) that this must represent the establishment of equilibria in which three or more components are present in appreciable amounts. In fact the anomalous mutarotation in such cases is often explained in terms of a pyranose-furanose interconversion. Apart from the furanose ring form, one of the components present in solution is probably the open chain aldehydo form which is usually postulated as the intermediary between the various pyranose and furanose forms. There is no direct proof of its existence, but Cantor & Peniston (1940) have shown that sugar solutions contain an isomer which is reducible at the dropping mercury electrode of the polarograph. The proportion of this reducible form is small ($<0{\cdot}2\%$) in the case of sugars which, according to mutarotation data, exist almost entirely in the pyranose form, but may be large with sugars such as D-altrose (1·1%) and D-ribose (8·5%) which exhibit complex mutarotations. In the arrangement of the results in Table 2 we have assumed that the sugars exist in solution almost entirely as the pyranose forms, though it is clear that in a number of cases the solutions must contain appreciable amounts of furanose and open chain aldehydo forms which will affect the observed R_F value.

EXPERIMENTAL

Many sugars used in this investigation were not available commercially and we are indebted to Prof. T. Reichstein, Pharmazeutisches Anstalt der Universität Basel, and Prof. C. S. Hudson, National Institute of Health, Bethesda, Maryland, for the gift of a number of the rarer sugars. These are marked in Table 2 with an asterisk and a dagger respectively. Of the remainder, most were synthesized by the epimerization in dry pyridine of the appropriate available sugar (Fischer, Taube & Baer, 1927; Danilow, Venus-Danilowa & Schantarowitsch, 1930). Normally two aldoses and a ketose are present in the mixture after epimerization which makes the chemical isolation of any particular sugar difficult. In the present study, however, this was not necessary as the mixture of sugars was separated on the paper chromatogram and the R_F value of the desired sugar measured at the same time. The various sugar spots were identified by spraying comparable papers (three was the maximum necessary) with reagents which reacted either with aldose or ketose sugars, and by comparison with such genuine specimens of the appropriate ketose and aldose sugars as were available. L-Gulose and L-idose produced by the epimerization of L-sorbose were identified after separation on the chromatogram by comparison with a genuine specimen of idose.

Aldoses. These gave a definite colour (red, green, brown, blue and yellow) if a paper was sprayed with 0·1 M-aniline hydrogen phthalate in moist *n*-butanol and then heated at 105° for 5 min. (Partridge, 1949). This reagent also reacts with ketoses if during the removal of the solvent used on the chromatogram they are heated with basic substances such as pyridine. Apparently some of the ketose is epimerized to aldose. As ethyl acetate-pyridine-water was used as the solvent for the majority of the observations in this study, the papers were dried at room temperature in a stream of air for at least 2 hr. to remove the pyridine before spraying with the aniline hydrogen phthalate. Under these conditions the reagent gave no reaction with ketoses.

Ketoses. These gave a coloration (red-brown or purple) if the paper was sprayed with a mixture of equal volumes of 0·2% (w/w) naphthoresorcinol in ethanol and 2% (w/w) trichloroacetic acid in water, and then heated at 105° for 5 min.

Aldoses and ketoses. The total number of sugar spots present was revealed by spraying a paper with 0·1 M-$AgNO_3$ in 5 M-NH_4OH and then heating at 105° for 5–20 min.

Methyl glycosides. These were prepared by dissolving the sugar (10 mg.) in 0·5% (w/w) HCl in methanol (1 ml.). Furanosides were produced by leaving the solution at room temperature for a period depending upon the sugar (2 hr. for pentoses and 15 hr. for hexoses) (Levene, Raymond & Dillon, 1932) and pyranosides by heating the solution at 70° for 3–4 hr. The solutions were then neutralized with Ag_2CO_3. The AgCl and excess Ag_2CO_3 were filtered off and the clear filtrates concentrated to about 0·3 ml. The mixture of glycosides was separated on the paper chromatogram and the R_F values of each individual glycoside measured at the same time. The position of the glycosides was revealed by spraying the paper with 0·1 M-$AgNO_3$ in 5 M-NH_4OH and then heating at 105° for 30 min. The paper darkened considerably, but the positions of the glycoside spots were easily visible. The silver oxide was reduced by the glycosides under these conditions. The free sugars present reduced the reagent within the first few minutes and could be distinguished readily from the glycosides which only reduced the reagent very slowly. The identification of furanoside and pyranoside was made on the assumption that the treatment at room temperature produced both furanoside and pyranoside, the furanoside usually in much greater amount, whereas the treatment at 70° produced only the pyranoside. Comparison of the glycoside spots produced by the two treatments indicated which was furanoside and which was pyranoside. In one case genuine methyl pyranosides were run on the paper at the same time. These were α- and β-methyl glucopyranoside. The result with these glycosides confirmed the previous identification of certain glucoside

spots as pyranose. There was a small difference between the R_F values of the α- and β-isomers.

A completely satisfactory determination of the R_F values of the methyl glycosides must await the provision of authentic specimens of each of the glycosides because, with the method described above, there is always some doubt about the identification of the various glycoside spots on the chromatogram. The doubt arises because it is known that a number of sugars can form derivatives with an anhydro ring in addition to the normal furanose or pyranose ring, and the presence of such derivatives will tend to confuse the identification of the various glycoside spots on the chromatogram. In certain cases the anhydro ring derivative is the main product of the action of acids on the free sugar. D-Altrose readily gives D-altrosan which is a 1:6-anhydro-D-altropyranose (Richtmeyer & Hudson, 1940). This carries only three hydroxyl groups and may be expected to have a higher R_F value than normal pyranosides.

The apparatus and the general procedure used for the accurate measurement of the R_F values was the same as that described earlier by Jermyn & Isherwood (1949). The measurements were carried out at a temperature of $20 \pm 0{\cdot}25°$. The solvent, which was the water-poor phase from a two-phase mixture of ethyl acetate (2 vol.), pyridine (1 vol.) and water (2 vol.), was freshly prepared for each run because it was found that the ethyl acetate present slowly hydrolysed. However, the change in composition in the first 1–2 days, the time necessary for the development of the chromatogram, had no appreciable effect on the R_F values. The mixed phases at the bottom of the chromatogram were also renewed each time because it was essential that the solvent on the paper and the liquid at the bottom of the jar were in equilibrium; any difference would tend to cause the composition of the solvent to change as it flowed down the paper.

The composition of the mixed phases at the bottom of the jar was also affected by the differential evaporation of the components to saturate the papers and the atmosphere of the jar in the preliminary equilibration. To minimize this effect at least 200 ml. of the mixed phases were added to a jar of the usual size (30–40 l.). The paper strip after the sugars had been applied was equilibrated for 24 hr. in contact with the mixed phases at the bottom of the jar before the solvent was allowed to run down the paper. For most solvents whose boiling points are about 100° this ensures not merely that the atmosphere in the chromatogram jar is saturated, but that the water-cellulose complex is in equilibrium with the solvent mixture. A simple experiment in which a paper (Whatman no. 1) was hung over a mixture of equal volumes of glacial acetic acid (b.p. 118°) and water in a chromatogram jar and the acetic acid present in equal areas of paper titrated after 24, 48 and 72 hr. showed that 4·3, 4·92 and 4·94 ml. of alkali were required respectively. Equilibration was practically complete after 48 hr. Using ethyl acetate-pyridine-water as solvent 24 hr. was sufficient. Recently Hanes & Isherwood (1949) have described a magnetic fanning device in which the papers are made to wave gently to and fro in the jar and thus stir the air. This greatly accelerates the process of equilibration so that a period of a few hours is sufficient.

Preliminary trials with D-glucose under the conditions described above indicated that the R_F values on the majority of papers varied by $\pm 0{\cdot}015$ about a mean value of 0·195 though some of the papers gave figures outside this range. Even this variation represents a difference between extremes of about 15%. It was essential to have a standard on each paper in order to determine whether the R_F values of the sugars on different papers could be compared and in practice D-glucose was used for this purpose. If the R_F value fell outside the range 0·20–0·19, the paper was rejected. No attempt was made to correct the results for variations in the R_F value of the D-glucose standard.

RESULTS

General relationship between the R_F value and the water content of the organic phase. In a previous paper Jermyn & Isherwood (1949) suggested that the R_F value of a sugar was related to the water content of the solvent, though no attempt was made

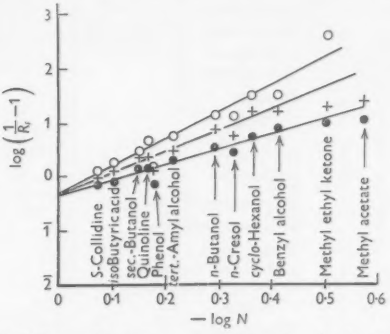


Fig. 1. Relationship between R_F value and molar fraction of water in non-aqueous phase. ○, Rhamnose; +, xylose; ●, glucose.

to formulate any definite relationship. In the present paper we have attempted to find a more precise relationship. By a re-examination of the data of Jermyn & Isherwood (1949) it has been found empirically that the graph of $\log([1/R_F]-1)$ against $-\log N$ gives a characteristic linear relationship for each sugar. N is the molar fraction of water in the solvent at 20°. In Fig. 1 the data for the three sugars rhamnose, xylose and glucose have been plotted. R_F values below 0·1 have not been included because a very small error in the measurement of the R_F value has a very large absolute effect on the value of $([1/R_F]-1)$. Plotting the data on a logarithmic scale has the advantage that the experimental errors in the measurement of the R_F value and the water content of the solvent do not assume too great a prominence at one end of the graph as compared with the other.

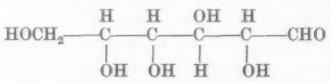
The results for phenol and *m*-cresol are particularly interesting because they do not agree with those obtained with other solvents. They give R_F values which are higher than would be expected from the amount of water dissolved in the organic solvent. It is possible that they can form loose compounds with the sugars which increase their solubility in the phenolic solvent.

Comparison between the configuration of the sugars and methyl glycosides and their R_F values in ethyl acetate-pyridine-water. A preliminary examination of the results indicated that if the sugars were arranged in groups on the basis of the ring structure assumed to exist in solution, the R_F values of corresponding members of each groups were similar. The mean figures for groups of sugars are given in Table 1. The individual results for most of the common sugars are given in Table 2.

Table 1. *Mean R_F values of various groups of sugars in ethyl acetate-pyridine-water at* 20°

Class of sugars	Ring form	Range of R_F values	Mean R_F value
Aldotetrose	Furanose	0·41 –0·43	0·42
Aldopentose	Pyranose	0·23 –0·33	0·285
Ketopentose	Furanose	0·35 –0·37	0·36
Aldohexose	Pyranose	0·175–0·31	0·24
Ketohexose	Pyranose	0·24 –0·30	0·26
Heptose	Pyranose	0·14 –0·23	0·18
Hexitol			
Sorbitol	.	0·18, 0·18,	0·19
Dulcitol	.	0·20	
Mannitol	.		
Aldohexomethylose	Pyranose	0·265–0·48	0·40
Ketohexomethylose	Furanose	0·43 –0·47	0·44
Disaccharide			
Sucrose, Trehalose	.	0·16, 0·15	0·13
Lactose, Cellobiose	.	0·07, 0·125,	
Maltose	.	0·13	
Trisaccharide			
Raffinose	.	.	0·044
Methyl glycosides of			
Aldopentoses	Pyranose	0·40 –0·48	0·44
	Furanose	0·52 –0·55	0·54
Methyl glycosides of			
Aldohexoses	Pyranose	0·23 –0·37	0·31
	Furanose	0·43 –0·48	0·46

The configuration of the sugars is described according to the Fischer convention, thus D-glucose



is written + + − +. The configuration of the ring is shown in the bracketed column, e.g. $\overbrace{+\,+\,-\,+}$.

For the purpose of description the carbon atoms of the ring are numbered from the reducing end and the numbers do not necessarily correspond with the usual numbering for the free sugar, thus D-fructopyranose would be described as having a $—CH_2OH$ group attached to carbon atom 1 of the pyranose ring, this carbon atom normally being regarded as number 2 of the sugar molecule.

In certain cases the D-sugars were not available and the results given are for the corresponding L-sugars. No difference between the R_F values for enantiomorphs in optically inactive solvents is likely. Even in optically active solvents no resolution of a DL mixture of sugars has been successful (Flood, Hirst & Jones, 1948).

A detailed comparison of the various sugars has been made in Table 3.

In Table 3(*a*) the R_F values of sugars which differ only in the disposition of the hydroxyl group on one carbon atom of the ring are compared so as to show the effect of changing one hydroxyl group on a particular carbon atom from above the ring to below the ring.

In Table 3(*b*) the R_F values of sugars which differ only in the disposition of the hydroxyl groups on carbon atoms 2 and 3 are compared. If the hydroxyl groups are on the same side of the ring, the arrangement is regarded as being *cis* and if on opposite sides as *trans*.

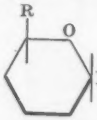
In Table 3(*c*) the effect of adding the group $—CH_2OH$ to either carbon atom 5 or 1 of the pyranose ring is studied by comparing aldohexoses and ketohexoses with aldopentoses which possess the same disposition of hydroxyl groups on carbon atoms 2, 3, 4 of the pyranose ring, irrespective of whether the sugar belongs to the L or the D series. In the case of the aldohexoses the sugars have been subdivided into two groups depending whether the group $—CH_2OH$ attached to carbon atom 5 is above the ring or below.

DISCUSSION

In a previous paper Jermyn & Isherwood (1949) suggest that the R_F values of the sugars run roughly parallel with the water content of the solvent and

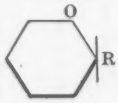
Table 2. *Relationship between the configuration and the R_F value of sugars and glycosides in ethyl acetate-pyridine-water at* 20°

Table 2. *Relationship between the configuration and the R_F value of sugars and glycosides in ethyl acetate-pyridine-water at 20°*

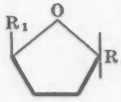
(*a*) Sugars with the general formula  **H.OH**, where R = —CH_3, —CH_2OH, —COOH, —CHOH.CH_2OH

Configuration (Ring)				Aldomethylose sugars			Aldohexose sugars			Aldohexuronic acids‡			Aldoheptose sugars		
				R = —CH_3	M.p. (°)	R_F	R = —CH_2OH	M.p. (°)	R_F	R = —COOH	M.p. (°)	R_F	R = —CHOH.CH_2OH	M.p. (°)	R_F
+	+	+	+	Allomethylose*	146	0·36	Allose*	128	0·22	.	.	.	.	.	.
+	+	+	−	Altromethylose	.	0·45	Altrose†	108	0·27	.	.	.	.	.	.
+	+	−	+	Glucomethylose	139–145	.	Glucose	146–150	0·195	Glucuronic	165	0·15	D-Gala-L-glucose†	190–194	0·16
+	−	+	+	Gulomethylose*	.	0·36	Gulose	.	0·23	.	.	.	D-Gluco-D-gulose†	210	0·19
+	+	−	−	Mannomethylose	123	0·38	Mannose	132	0·24	Mannuronic	165	0·18	D-Gala-L-mannose†	.	0·21
+	−	+	−	Idomethylose*	.	0·51	Idose	.	0·31	.	.	.	.	.	.
+	−	−	+	Galactomethylose	145	0·265	Galactose	165	0·175	Galacturonic	160	0·13	D-Gulo-L-galactose†	.	0·14
													D-Manno-D-galactose†	134	0·15
+	−	−	−	Talomethylose*	.	0·48	Talose*	128	0·285	.	.	.	D-Gulo-L-talose†	.	0·23

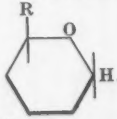
Sugars marked * were a gift from Prof. T. Reichstein and those marked † were from Prof. C. S. Hudson.
‡ These were run in ethyl acetate-acetic acid-water as solvent (Jermyn & Isherwood, 1949).

(*b*) Sugars with the general formula  **R.OH**, where R = —H, CH_2OH

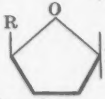
Configuration (ring)			Ketohexose sugars			Aldo-pentose sugars		
			R = —CH_2OH	M.p. (°)	R_F	R = —H	M.p. (°)	R_F
+	+	+	Allulose	Syrup	0·30	Ribose	95	0·33
+	−	−	Tagatose	124	0·27	Lyxose	106–117	0·30
+	−	+	Sorbose	164	0·24	Xylose	145	0·28
+	+	−	Fructose	102	0·24	Arabinose	159	0·23

(*c*) Sugars with the general formula  **R.OH**, where R = —H, —CH_2OH and R_1 = —H, —CH_3

Configuration (ring)		Aldotetrose sugars		Ketopentose sugars		Keto-hexomethylose sugars	
		$R = R_1$ = —H	R_F	$R = R_1$ = —CH_2OH	R_F	R_1 = —CH_3 R = —CH_2OH	R_F
+	+	Erythrose	0·41	Araboketose	0·35	Allomethylo-ketose + + +	0·44
						Galactomethylo-ketose + − −	0·43
+	−	Threose	0·43	Xyloketose	0·37	Mannomethylo-ketose + + −	0·43
						Idomethylo-ketose + − +	0·47

(*d*) Glycosides with the general formula  **H.OCH_3**, where R = —H, —CH_2OH

Configuration (Ring)				Aldohexose glycosides		Aldopentose glycosides	
				R = —CH_2OH	R_F	R = —H	R_F
+	+	+	+	Allose	0·23	Ribose	0·40
+	+	+	−	Altrose	0·36	Arabinose	.
+	+	−	+	Glucose	0·29	Xylose	0·46
+	−	+	+	Gulose	.	.	.
+	+	−	−	Mannose	0·37	Lyxose	0·48
+	−	+	−	Idose	.	.	.
+	−	−	+	Galactose	0·34	.	.
+	−	−	−	Talose	0·29	.	.

(*e*) Glycosides with the general formula  **H.OCH_3**, where R = —CH_2OH, —CHOH.CH_2OH

Configuration (Ring)				Aldohexose glycosides		Aldopentose glycosides	
				R = —CHOH.CH_2OH	R_F	R = —CH_2OH	R_F
+	+	+		Allose	0·45	Ribose	0·54
+	+	−		Altrose	0·48	Arabinose	0·55
+	−	+		Glucose	0·43	Xylose	0·52
−	+	+		Gulose	.	.	.
+	−	−		Mannose	0·48	Lyxose	0·55
−	+	−		Idose	.	.	.
−	−	+		Galactose	0·47	.	.
−	−	−		Talose	0·44	.	.

Table 3. *Influence of the configuration of the hydroxyls on each carbon atom of the pyranose ring on the R_F values of the sugars*

(*a*) Comparison of sugars in which the hydroxyl on one carbon atom has been changed from above to below the ring

Hydroxyl inverted on carbon atom no.	Sugars compared	Difference in R_F value
4	Gulose, allose	+0·01
	Idose, altrose	+0·04
	Galactose, glucose	−0·02
	Talose, mannose	+0·055
3	Glucose, allose	−0·025
	Mannose, altrose	−0·03
	Galactose, gulose	−0·055
	Talose, idose	−0·025
2	Altrose, allose	+0·05
	Mannose, glucose	+0·045
	Idose, gulose	+0·08
	Talose, galactose	+0·11
3	Xylose, ribose	−0·05
	Lyxose, arabinose	+0·07
2	Arabinose, ribose	−0·10
	Lyxose, xylose	+0·02

(*b*) Interaction of hydroxyls on adjacent carbon atoms

Sugar	Configuration of hydroxyls on carbon atoms 3	2		R_F	Sugar	Configuration of hydroxyls on carbon atoms 4	3	2		R_F
Ribose	+	+	*cis*	0·33	Allose	+	+	+	*cis*	0·22
					Altrose	+	+	−	*trans*	0·27
Arabinose	+	−	*trans*	0·23	Glucose	+	−	+	*trans*	0·195
					Mannose	+	−	−	*cis*	0·24
Xylose	+	−	*trans*	0·28	Idose	−	+	−	*trans*	0·31
					Gulose	−	+	+	*cis*	0·23
Lyxose	−	−	*cis*	0·30	Galactose	−	−	+	*trans*	0·175
					Talose	−	−	−	*cis*	0·285

(*c*) The effect of adding the group —CH_2OH to either carbon atom 1 or 5 of the pyranose ring

Configuration of ring			Aldohexoses —CH_2OH on carbon atom 5	R_F		R_F	Ketohexoses —CH_2OH on carbon atom 1	R_F	Aldopentoses No substituent	R_F
+	+	+	Allose	0·22	Talose	0·285	Allulose	0·30	Ribose	0·33
+	−	−	Mannose	0·24	Gulose	0·23	Tagatose	0·27	Lyxose	0·30
+	−	+	Glucose	0·195	Idose	0·31	Sorbose	0·24	Xylose	0·28
+	+	−	Altrose	0·27	Galactose	0·175	Fructose	0·24	Arabinose	0·23

that the order in which the sugars separate is not affected by the composition of the solvent. In the present paper this suggestion has been investigated more thoroughly and the results given in the earlier paper have been interpreted so as to show in a more striking manner the exact relation which exists between the movement of the sugars on the chromatogram and the water content of the solvent. The water content of the solvent (N) is defined as the molar fraction of water in the non-aqueous phase at 20°. It has been found empirically that if $\log([1/R_F]-1)$ is plotted against $-\log N$ for each sugar then the graph is a straight line. Examples of this are shown in Fig. 1, for the three sugars L-rhamnose, D-xylose and D-glucose. The graphs show clearly that the value of $([1/R_F]-1)$, which can be regarded as equivalent to the distribution of the sugar between the water-cellulose complex and the solvent, cf. equation (1), is governed almost entirely by the water content of the solvent. The graphs also show that the relative values of $([1/R_F]-1)$ for the various sugars remain in the same order independent of the water content of the solvent. The value of this general relationship be-

tween the movement of the sugars on the chromatogram and the water content of the solvent is that the results of a detailed study in one particular solvent can now be applied to a variety of solvents. The order of R_F values will be similar in each solvent except phenols which will be referred to later. However, it should be pointed out that this generalization, so far as this study is concerned, applies to a temperature of about 20° and solvents which are the water-poor phase of a two-phase mixture.

A plausible theoretical basis for the empirical relationship described above can be deduced from the fact that sugars in solution have a strong affinity for water molecules which are presumably bound to the hydroxyl groups of the sugars. The sugars in aqueous solution are in fact very heavily hydrated and may be expected to approximate in behaviour to water itself. The affinity of sugars for neutral organic molecules is small, and it seems that it is the heavily hydrated sugar molecules which are soluble in the aqueous organic solvents. It follows, therefore, that the distribution of a sugar between an aqueous solvent and the water-cellulose complex will depend to a large extent on the readily available water in the two phases. It is probable, however, that the water bound to the cellulose which is available to the sugar on the chromatogram, is only slightly affected by changes in the composition of the solvent so that the distribution of a sugar between the two phases is largely controlled by the water content of the solvent. If one regards both the solubility of the water and the heavily hydrated sugar as manifestations of the affinity of molecules of the solvent for water hydroxyls, then the distribution of the sugar between the two phases may be expected to run parallel with the solubility of water in the organic phase.

In the present paper the R_F values of the sugars were measured using the water-poor phase of a two-phase mixture of ethyl acetate-pyridine-water. Accurate R_F values for a number of the sugars using this solvent had already been obtained before this study was started and as the solvent was readily available and convenient to use, it was adopted as standard.

In Fig. 1 it will be noticed that the results for phenol and *m*-cresol are not consistent with those obtained with other solvents, the R_F values being greater than would be expected from the water content. In addition it has been found that the order in which the sugars separate on the chromatogram is different, thus the order of R_F values in phenol and in ethyl acetate-pyridine-water is glucose, galactose, mannose and galactose, glucose, mannose respectively. The explanation probably lies in the fact that phenols can form compounds with sugars analogous to those formed by water so that the distribution of a sugar between the aqueous phenolic solvent and the water-cellulose complex will depend not only on the water present in each phase but also on the phenol. However, apart from phenols, the order in which the sugars separate on the paper chromatogram is independent of the solvent.

In Table 1 the mean R_F values for various groups of sugars are listed. The comparable figures for the methyl glycosides and for the di- and tri-saccharides are also given. A brief examination of the figures indicates that while the number of hydroxyl groups in a monosaccharide has some relation to the R_F value, other factors contribute largely to the observed results. The mean R_F values for the hexo-methyloses (0·40 and 0·44) and pentoses (0·29 and 0·36) and for the methyl pyranosides (0·44 and 0·31) and furanosides (0·54 and 0·46), show that even if the number of hydroxyl groups is the same, the R_F values may be very different. Grouping the sugars on the basis of the number of hydroxyls alone, would mean in the case of the pentoses, hexoses and heptoses, that the range of R_F values in each group considerably overlapped those in the other groups. Comparison of the mean R_F values for the mono-, di-, and tri-saccharides illustrates the effect of varying the number of sugar residues in the molecule.

In Table 2 the sugars have been arranged in groups on the basis of the configuration of the atoms which compose their ring forms. Thus the pyranose forms of D-xylose, D-glucose, D-glucomethylose, and L-gala-D-glucoheptose all belong to the D-glucose type. These sugars differ only in the group attached to carbon atom 5 of the pyranose ring, the configuration of the atoms of the ring being the same in each case. Such a grouping of sugars has been termed homomorphous (Hann, Merrill & Hudson, 1935), and as would be expected from the identity of the configurations of the rings, members of each homomorphous series often show marked chemical and physical similarities with each other. In such a series it is often possible to predict the properties of an unknown member from those of the basic type. In the present study the ring structures were assumed to be pyranose, except where the chemical structure of the sugar prevented this being formed. Thus, the ketopentoses and aldotetroses were formulated as furanose.

Comparison between the various groups in Table 2 shows that the R_F values of corresponding members of each group are markedly dependent on the configuration of the ring. In addition the residue attached to either carbon atom 5 of the pyranose ring or 4 of the furanose ring exercises the same general influence on the R_F value independent of the configuration of the ring. This means that the same sequence of R_F values is preserved in each of the homomorphous groups. Comparison of the sugars in Table 2(*a*) shows that the replacement of

—CH_2OH of any of the aldohexose sugars by —CH_3 or —$CHOH.CH_2OH$ changes the R_F value by about +50 and −15% respectively. A similar effect is observed with the residue attached to the reducing end of the ring. Comparison of the ketohexose and aldopentose sugars in Table 2(*b*) shows that the replacement of —H on carbon atom 1 of any of the aldopentose sugars by —CH_2OH changes the R_F value by about −10%. The sugars which can only form furanose rings have much higher R_F values than those which can form pyranose, and the differences between individual sugars are less. The influence of the ring form is very clearly demonstrated in the case of the aldohexose methyl pyranosides and furanosides. Reference to Table 2(*d*) and (*e*) shows that the range of R_F values for the pyranosides 0·23–0·37 is wider, and the mean value 0·31 smaller, than for the corresponding furanosides (0·43–0·48 and 0·46 respectively).

Comparison of the various homomorphous groups is probably helped by the fact that the influence of the configuration of the ring on other properties such as the proportion of the various ring isomers in solution is automatically brought into line with the R_F values. Sugars which have the same configuration for the first four carbon atoms will give very similar mixtures of the various ring forms. Evidence on this point is rather scanty, but it is known that D-altrose (++++) and D-ribose (+++) are characterized in solution by the presence of large amounts of the aldehydo forms, approximately twenty times that of other sugars in their class. This fact suggests that the pyranose ring with the configuration (+++) is rather unstable.

In Table 2 we have included the melting points of a number of the sugars for comparison with their R_F values. In the case of the aldopentoses and aldohexoses the melting point is roughly inversely proportional to the R_F value. Talose is exceptional in that its melting point is somewhat higher than the other sugars with a similar R_F value. In the case of other classes of sugars the melting point does not correspond in any simple manner to the R_F value; of the ketohexose sugars, allulose, tagatose and sorbose behave like the aldohexoses, but fructose is very different. From its position in the table it ought to have a melting point higher than 164° and an R_F value of less than 0·24, whereas it has a melting point of 102° and an R_F value of 0·24. The important fact about the comparison between the melting point and the R_F value is that a close relationship does exist within a restricted group of sugars. This is sufficiently remarkable to stimulate speculation as to what fundamental property of the sugar molecule forms the link between the melting point and the R_F value. The melting point of an organic crystal is a measure of the strength of the internal bonds between the molecules in the lattice and in the case of the sugars which are mainly linked together by their hydroxyl groups, must represent at least approximately the extent and strength of the association of these hydroxyl groups. The R_F value, as described previously, is a measure of the affinity of a sugar for water, the affinity being inversely proportional to the R_F value. In solution the sugar molecules are largely hydrated. Now Cox *et al.* (1935) have pointed out that for certain common pentoses and hexoses (figures were not available for any others) the molecular volume in the crystalline state is very similar to the aqueous solution volume which they suggest means that the association of a sugar with water molecules in solution is of the same nature and extent as the association of neighbouring sugar molecules in the solid. We may expect, therefore, the affinity of a sugar for water molecules to be closely related to the strength of the internal bonds between the sugar molecules in the crystal and correspondingly the R_F value to be inversely proportional to the melting point. However, any discrepancy between the form of the molecule in the solid state and in solution will cause the relationship to break down. It is perhaps significant that the sugars for which figures were available of the molecular volume in the crystal state and in solution (Cox *et al.* 1935) were aldopentoses and aldohexoses. These give the simple relationship between the melting point and the R_F value. The examination of the other classes of sugars which do not give this relationship might show considerable discrepancies between the form of the molecule in the solid state and in solution.

A more detailed comparison of the various sugars has shown that in many cases the R_F value can be related even more closely to the configuration of the ring. In Table 3(*a*) the R_F values of sugars which differ only in the disposition of the hydroxyl group on one carbon atom of the ring are compared so as to show the effect of changing one hydroxyl group on a particular carbon atom from above the ring to below the ring. In the case of the aldohexoses, the figures for the various pairs of sugars, with the exception of galactose-glucose, show that the change alters the R_F value in the same sense for any one particular carbon atom, but that the direction in which the R_F value is altered is different for each carbon atom. In the case of the aldopentoses, no clear relationship is discernible between the R_F value and the disposition of the hydroxyl groups above or below the ring. In Table 3(*b*) the R_F values of sugars which differ only in the disposition of the hydroxyl groups on carbon atoms 2 and 3 are compared. If the hydroxyl groups are on the same side of the ring, the arrangement is regarded as being *cis* and if on opposite sides *trans*. In the case of the aldopentoses the figures indicate that the *cis* arrangement has a higher R_F value than the *trans*, but that in the case of the aldohexoses no such simple relationship exists.

Confirmatory evidence of the significance of the relationship in the case of the aldopentoses which are admittedly a small sugar group, is provided by a study of the ketohexoses, Table 3(*c*). The order of R_F values corresponds to that expected from analogy with the aldopentoses, so that in this case also the *cis* arrangement has a higher R_F value than the *trans*. The effect of the configuration of the hydroxyl groups on carbon atoms 3 and 4 has also been examined, but the most careful scrutiny has not shown any sign of regularity. In Table 3(*c*) the effect of adding the group —CH_2OH to either carbon atom 5 or 1 of the pyranose ring is studied by comparing aldohexoses and ketohexoses with aldopentoses which possess the same disposition of hydroxyl groups on carbon atoms 2, 3, 4 of the pyranose ring. It is noticeable that the order of R_F values for the first group of aldohexoses (—CH_2OH above the ring) is the exact opposite of that for the second (—CH_2OH below ring). This suggests that the disposition of the —CH_2OH group on carbon atom 5 must have a very strong influence on the R_F value. The comparison between the aldohexoses, ketohexoses and aldopentoses indicates that adding the group —CH_2OH to carbon atom 5 completely changes the influence of the ring hydroxyl groups on the R_F value whereas adding it to carbon atom 1 has very little effect.

The overall picture obtained from the results in Table 3 is that the presence of a substituent on carbon atom 5 of the pyranose ring appears to have a profound influence on the interaction between the various hydroxyl groups attached to the ring and on the affinity of the sugar for water molecules. In the absence of a substituent, the association of a sugar with water molecules is limited only by the interaction between the hydroxyls on neighbouring carbon atoms. In the presence of a substituent, the association of a sugar with water molecules is governed by whether the hydroxyl groups are on the same side of the ring as the substituent or not. The fact that the order of R_F values for the methylose sugars is the same as for the corresponding aldohexoses indicates that the group —CH_3 influences the R_F value in the same general manner as —CH_2OH and suggests that the substituent does not act through an association of hydroxyl groups between carbon atom 6 and the ring, but rather through some form of steric hindrance. This interference with the interaction of the hydroxyl groups of the pyranose ring with each other and with water molecules disappears if the substituent is attached to carbon atom 1 instead of carbon atom 5. Comparison of the ketohexoses in which the group —CH_2OH is attached to carbon atom 1 of the pyranose ring, and the aldopentoses, shows that the order of R_F values in each group of sugars corresponds very closely. This implies that the group —CH_2OH has very little effect on the interaction of the hydroxyl groups of the pyranose ring which are the controlling influence in the case of the aldopentoses. It is possible, however, that in solution the ring form of the ketohexoses adjusts itself so as to eliminate largely any steric hindrance, an adjustment which is probably impossible when the group —CH_2OH is attached to carbon atom 5 of the pyranose ring. It is noteworthy that in solutions of the ketohexoses considerable amounts of the reducing open chain forms are present and that in the case of fructose (Gottschalk, 1943) about 20 % of the sugar exists as the furanose form. These observations suggest that the pyranose ring is unstable with the substituent on carbon atom 1 of the ring and it may be that a steric factor is responsible.

The furanose sugars have not been examined in the same detail as the pyranose sugars because it is apparent from a brief inspection of the results in Table 2(*c*) that the configuration of the hydroxyl groups on the ring has less influence on the R_F value. In Table 2(*c*) the sugars have been grouped so as to bring out similarities in R_F value for similar types of ring structure. Thus D-galacto-methylo-ketose $+--$ has been compared with D-ethythrose $++$. Strictly L-galacto-methylo-ketose $-++$ should be compared.

The R_F values for the glycosides given in Table 2(*d*) and (*e*) probably represent the mean values for the α- and β-isomers. In many cases the amount of sugar at our disposal was too small to enable us to make a detailed investigation and to that extent the results must be regarded as provisional. The R_F values for genuine specimens of α- and β-methyl-gluco-pyranoside (0·29 and 0·305) were sufficiently close to suggest that no separation of the αβ-isomers would occur on the paper chromatogram using ethyl acetate-pyridine-water as solvent. The results for the methyl glycosides have been arranged on the basis of the configuration of the ring in a similar manner to the sugars. It is not our intention to analyse these results in detail owing to possible uncertainties in some of the results. However, comparison between the various groups shows that the R_F values of corresponding members of each group are markedly dependent on the configuration of the ring. In addition, it is significant that the order of R_F values for the methyl pyranosides is not the same as that for the parent sugars. D-Galactose which has the lowest R_F value of the aldohexoses gives a methyl pyranoside which has a R_F value well above the lowest of its class. Furanosides have much higher R_F values than pyranosides.

SUMMARY

1. An empirical relationship has been discovered between the movement of the sugars (R_F value) on the paper chromatogram and the molar fraction of water (N) in the solvent. The graph of log $([1/R_F]-1)$

against $-\log N$ is a straight line for each sugar. The relationship holds over a wide range of solvent mixtures, the only exceptions being those containing phenols as the organic component. The relationship is given a theoretical basis in terms of the strong association of the hydroxyl groups of the sugars with the water molecules in mixed solvents containing water. The sugars separate in the same order in all solvents, except phenols.

2. If the sugars are arranged in the form of a homomorphous series on the basis of an assumed preferential formation of a pyranose ring in solution (furanose when pyranose is impossible) then it is found that members of each homomorphous series show close similarity in behaviour on the paper chromatogram. The sequence of R_F values for each group of sugars (aldohexose, aldomethylose, etc.) depends only on the configuration of the hydroxyls of the ring. The same appears to be true of the methyl glycosides.

3. A detailed analysis has been made of the contribution of each hydroxyl group to the observed R_F value in the case of the aldohexoses, aldopentoses and ketohexoses. The analysis shows that if a substituent (—CH_2OH) is attached to carbon atom 5 of the pyranose ring then the influence of a hydroxyl group on any particular carbon atom largely depends upon whether the hydroxyl is on the same side of the ring as the substituent or not. The effect on the R_F value is different for each carbon atom. In the absence of a substituent (aldopentoses) or if the substituent is attached to carbon atom 1 of the pyranose ring (ketohexoses), it is the interaction between the hydroxyl groups on neighbouring carbon atoms which governs the R_F value, a *cis* disposition of hydroxyls giving a higher R_F value than a *trans*.

4. Sugars with a furanose ring have a much higher R_F value than those with a pyranose ring.

5. The R_F values of the simple pentoses and hexoses are roughly inversely proportional to their melting points.

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The Mechanism of the Formation of Organic Acids by Mould Fungi

5. THE INFLUENCE OF ARSENITE ON *ASPERGILLUS NIGER*

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Chughtai, Pearce & Walker (1950) showed recently that acetic acid is produced in the mycelial felts of cultures of *Aspergillus niger* growing on a glucose medium and that, following the addition of sodium arsenite to such cultures, pyruvic acid may be detected in the medium. A quantitative study of the effects of arsenite on the utilization of sugar and on the formation of acids in this mould has now been carried out.

EXPERIMENTAL METHODS AND RESULTS

The strain of *A. niger* (designated N1) used generally in previous studies in this series was again employed. The medium in the quantitative experiments consisted of

glucose (10%, w/v) in an aqueous solution containing (w/v) NH_4NO_3, 0·2%; KH_2PO_4, 0·1%; and $MgSO_4.7H_2O$, 0·05%. The glucose was sterilized separately in 40% solution (w/v) in distilled water and measured volumes of this solution were added aseptically to the flasks containing the sterilized solution of salts.

The procedure of Willstätter & Schudel (1918) was employed for estimation of glucose. Citric, oxalic and gluconic acids were estimated by methods described by Butkevich (1922, 1924).

In a preliminary experiment it was ascertained that spores of the mould could develop normally in presence of sodium arsenite at concentrations of the latter varying from 0·001 to 0·004 M. At 0·004 M the rates of sugar consumption and of acid formation were both greater than in the control. Arsenite at 0·010 M inhibited the germination of spores.

In another experiment the mould was grown at 30° from spores sown on 3780 ml. of the medium contained in twenty-seven 600 ml. conical flasks, which had wide necks. The mycelial felts being well developed at 3 days, six of the felts were removed, washed with water, drained, dried at 70° and weighed. The medium in the twenty-one remaining flasks was drained away from under the felts and replaced by sterile salt solution without glucose, the flasks being then left at 30° overnight. Next morning, the liquid contents were poured away and replaced in six of the flasks (the control) by a sterile 10% (w/v) aqueous solution of glucose, and in the other flasks by a 10% aqueous solution of glucose containing sodium arsenite at concentrations of 0·001, 0·002, 0·004, 0·010 or 0·020 M, there being thee flasks for each concentration of arsenite. The volume of glucose solution or of glucose solution containing arsenite, which

Table 1. *Consumption of glucose and formation of citric, oxalic and gluconic acids by* Aspergillus niger *in presence and in absence of sodium arsenite*

(Glucose present initially at a concn. of 8·92 g./100 ml., equivalent to 3·5 g. carbon/100 ml.)

				Acid formation (g. carbon)					
				Citric		Oxalic		Gluconic	
Arsenite concn. (M)	Time (days)	Glucose utilized (g. carbon/ 100 ml.)	Acidity (ml. 0·1N-NaOH/ 100 ml.)	(g./100 ml.)	(% yield)*	(g./100 ml.)	(% yield)	(g./100 ml.)	(% yield)
Control (no arsenite	2	0·328	68	0·121	37·0	Nil	—	0·122	37·3
	4	0·764	124	0·196	25·7	0·011	1·4	0·237	31·1
	6	1·420	176	0·295	20·8	0·029	2·0	0·208	14·7
0·001	2	0·380	94	0·157	41·0	0·027	7·2	0·036	9·4
	4	0·876	154	0·209	23·9	0·053	6·0	0·165	19·0
	6	1·564	184	0·247	15·8	0·047	3·0	0·302	19·3
0·002	2	0·400	60	0·074	18·5	Trace	—	0·208	52·2
	4	1·528	310	0·612	40·1	0·040	2·6	0·158	10·4
	6	2·476	376	0·647	26·1	0·050	2·0	0·460	18·6
0·004	2	0·380	48	0·014	3·8	Trace	—	0·302	78·7
	4	1·856	462	0·804	43·3	0·032	1·7	0·719	38·8
	6	2·552	606	1·016	37·2	0·075	2·8	0·863	31·7
0·010	2	0·200	24	Trace	—	Nil	—	—	—
	4	0·912	320	0·470	49·5	0·096	10·1	0·295	31·2
	6	1·460	386	0·530	37·1	0·150	10·3	0·287	19·8
0·020	2	0·128	20	Nil	—	Nil	—	Nil	—
	4	0·144	27	Nil	—	Nil	—	Nil	—
	6	0·216	63	Trace	—	Nil	—	Nil	—

* Percentage yield on glucose utilized.

Table 2. *Influence of sodium arsenite on the weight of mycelium developed and on the quantity of* CO_2 *evolved by cultures of* Aspergillus niger *in a glucose medium*

(Carbon content of felt at 3 days, prior to introduction of arsenite = 0·362 ± 0·018 g./flask.)

			Gain in carbon in felt in last 3 days		Sum of citric, oxalic and gluconic acids plus gain in felt	CO_2 evolved, by calculation	
Arsenite concn. (M)	Glucose utilized (g. carbon/ 157 ml.)	Carbon in felt at 6 days (g./157 ml.)	(g./157 ml.)	(% yield)*	(g. carbon/ 157 ml.)	(g. carbon/ 157 ml.)	(% yield)
Control (no arsenite)	1·988	0·897	0·535	33·6	1·279	0·709	35·7
0·001	2·189	1·072	0·710	32·2	1·544	0·645	29·5
0·002	3·466	1·115	0·752	21·7	2·372	1·094	31·5
0·004	3·573	1·255	0·892	25·0	3·627	−0·054	−1·0†
0·010	2·044	0·605	0·242	11·8	1·596	0·448	21·4
0·020	0·302	0·352	—	—	—	—	—

* Percentage yield on glucose utilized. † Signifies a slight uptake of atmospheric CO_2.

was so added, was in all cases 140 ml./flask. However, since the washed felts retained salt solution in their under-surface convolutions, a correction became necessary, for preliminary tests had shown that such interstitial solution, amounting to 17 ml./flask, reduced the concentration of glucose from 10 to 8·92 % (w/v), and subsequent calculations of yields of products were therefore based on the latter figure. All the flasks were returned to the incubator (30°) and at further intervals of 2, 4 and 6 days a sample of the medium was withdrawn from each flask, for analysis. At the end of 6 days all the felts were removed, washed with water, drained, dried at 70° and weighed.

The results have been collected in Tables 1 and 2. Theoretically, 180 g. of glucose could furnish as a maximum yield, 192 g. of citric acid, or 270 g. of oxalic acid, or 196 g. of gluconic acid, and the yields of these three acids have been calculated on the basis of these figures. The quantities of sugar, of acids, of carbon dioxide and of mould felt have, in all cases, been expressed in terms of g. carbon; in Table 1, as g. carbon/100 ml. of medium and in Table 2 as g. carbon/157 ml. of medium. The latter table shows increases in the weights of the felts which had of necessity to be determined on whole felts, each developed in 157 ml. of 8·92 % (w/v) glucose medium. The percentage of carbon in the mould felt was determined by the wet combustion method of Hockenhull (1950) and showed that $50 \pm 0{\cdot}5$ % of the increase in weight of felt during the last 3 days of incubation was represented by carbon. The synthesis of additional protein during this period could not have accounted for an appreciable share of this increase in weight, because the original medium containing inorganic nitrogen had been poured away at the end of 3 days and replaced by the nitrogen-free glucose medium. The value 50 % justifies the inference that the gain in weight was due principally to accumulation of reserve polysaccharide and, to a much less extent, to the formation of fat. In the last column of Table 2 the carbon dioxide evolved is shown as a percentage on the weight of glucose utilized. The actual output of carbon dioxide was not measured and the figures in this column have therefore been ascertained by calculation.

DISCUSSION

Inhibition of respiration without interference with alcoholic fermentation in yeast cells treated with sodium arsenite at certain concentrations was observed by Szent-Györgyi (1930). On the assumption that citrate formation in *Aspergillus niger* depends upon a supply of pyruvate furnished by the processes of alcoholic fermentation (a view in favour of which experimental evidence can be adduced, Walker, 1949) and also on the assumption that arsenite produces in *A. niger* effects similar to those which it causes in yeast, it would be expected that in cells of this mould, in presence of arsenite, the balance of enzymic activities would be deflected from oxidation processes ending in evolution of carbon dioxide and diverted towards citrate formation. This, in fact, is what happens, as is clearly shown by the present results. The effects of arsenite are not merely limited, however, to switching this reaction, but are evidently more profound, as is evidenced by the considerable ncrease in general metabolic activity seen in the presence of arsenite at certain levels. Thus, young preformed felts of the mould treated with arsenite at 0·004 M were especially active, sugar consumption in 6 days being approximately double that in the control, while during the same period, the weights of citric, oxalic and gluconic acids produced were, respectively, 3·4, 2·6 and 4·1 times those produced in the control. Arsenite at 0·004 M also completely prevented carbon dioxide output and promoted the greatest synthesis of felt.

At 0·010 M-arsenite the weight of citric acid formed was nearly double that in the control, although the quantity of glucose utilized in 6 days (about 40 %) was practically the same in the two cases. At 0·010 M-arsenite the weight of gluconic acid formed was still higher than in the control, while oxalate formation, though not high, was relatively much greater than at lower concentrations of arsenite. Accumulation of felt was depressed, however, at 0·010 M-arsenite. Krebs (1933*a*, *b*) and Krebs & Johnson (1937) established the fact that arsenite is a specific inhibitor of the oxidative decarboxylation of certain α-ketoacids in animal tissues. If it were known that the tricarboxylic acid cycle proceeds in *A. niger* it would be obvious that both the enhancement of yield of citric acid and the reduction in the output of carbon dioxide could be explained by the stabilizing effect of arsenite on α-ketoglutaric acid. However, we have as yet no direct experimental proof that this cycle operates in moulds which form citric acid, and the very high yields of the latter (about 85 %) which some strains produce are, in fact, evidence against the probability of the occurrence of this cycle in their cells. It has been shown, however, by Chughtai *et al.* (1950), that arsenite stabilizes pyruvic acid in cultures of *A. niger* on a glucose medium, and this effect may result in a greater proportion of the pyruvic acid becoming converted, by carbon dioxide fixation, to oxaloacetic acid, leaving proportionally less pyruvic acid to be converted to acetic acid. Should arsenite thus displace within certain limits the ratio of the products derived immediately from pyruvic acid, the final effects would be those observed in our experiments, namely, increases both in formation of citric acid and in conservation of carbon dioxide.

Finally, the reasons for the stimulating effects of arsenite on the formation of oxalic and gluconic acids must remain topics for further study, since the present data do not throw light on these questions.

SUMMARY

1. Spores of *Aspergillus niger* in a glucose medium developed normally in presence of sodium arsenite at concentrations varying from 0·001 to 0·004 M, but germination was inhibited at 0·010 M.

2. Preformed mycelial felts of *A. niger* treated with arsenite at concentrations 0·001, 0·002, and 0·004 M, respectively, subsequently consumed more sugar, accumulated more felt material and produced a greater total of acids (citric, oxalic and gluconic) in a given time than did controls in absence of arsenite. Enhancement of metabolic activity was greatest at 0·004 M.

3. In presence of 0·010 M arsenite sugar consumption by preformed felts was not appreciably greater than it was in absence of arsenite, while increase in felt weight was less than in the arsenite-free control. Formation of acids in presence of 0·010 M-arsenite was greater than in the control, but less than it was at 0·004 M. Arsenite at 0·020 M almost completely suppressed the mould's activities.

4. Arsenite at all concentrations between 0·001 and 0·010 M reduced carbon dioxide output. This effect was greatest at 0·004 M.

5. The bearing of these results on the mode of formation of citric acid is discussed.

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Studies on the Biochemistry of Zinc

1. EFFECT OF FEEDING ZINC ON THE LIVER AND BONES OF RATS

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The indispensability of zinc as an element in nutrition has now been fairly established. Todd, Elvehjem & Hart (1934), Stirn, Elvehjem & Hart (1935), Day & McCollum (1940) and Day & Skidmore (1947) were able to show that when animals were fed a diet deficient in zinc they grew subnormally and developed various symptoms which were cured by supplementing the diet with small amounts of zinc, but attempts to elúcidate the mechanism of its action have not been very successful. Hove, Elvehjem & Hart (1937) showed that deficiency of zinc resulted in the impairment of intestinal absorption. Other changes brought about by a deficiency of this element have been reported by Gorter (1939) and Day (1942). Scott (1934) and Scott & Fisher (1935, 1938*a*, *b*) showed that even the purest preparations of insulin contain zinc and that this element is somehow associated with the hypoglycaemic action of insulin. Keilin & Mann (1939) reported the presence of this element as a component of the enzyme, carbonic anhydrase, but Hove, Elvehjem & Hart (1940*a*, *b*) and Day & McCollum (1940) could find no change in the carbonic anhydrase activity in cases of zinc deficiency. Davidson (1942) and Holmberg (1939) reported the presence of zinc in their preparations of uricase, but Wachtel, Hove, Elvehjem & Hart (1941) could not observe any difference in the uric acid content of the blood of normal and deficient animals. Further work is therefore necessary to find out how this element functions as an indispensable constituent of food of animals.

In this and the following papers, the problem has been attacked from a standpoint somewhat different from that of the investigations mentioned above. Here, the changes brought about by zinc when fed in concentrations much higher than the optimum have been investigated since it was observed in the preliminary experiments of the author that in broad outline, the changes brought about by either a deficiency of zinc or by toxic concentrations of it were almost identical. Moreover, since the preparation of diets completely deficient in this element without affecting any of the other constituents in the diet is very laborious and not quite satisfactory, it was thought that the changes brought about by the element when fed in toxic concentrations would be easier to follow and would lead eventually to a better elucidation of the biochemical functions of

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this element. The process may be regarded as analogous to the elucidation of the nature of activity of an enzyme by an investigation of its activation and inhibition.

Investigations on the changes brought about by diets containing excess zinc have been few. Grimmett, McIntosh, Wall & Hopkirk (1937) reported that, on a high-zinc diet, pigs showed toxic symptoms in about 2 months. This toxicity syndrome was characterized by loss of appetite and stiffness of the legs. On autopsy, the liver showed necrosis and the head of the humerus was affected. They also reported that zinc accumulated in the pancreas, kidney, liver and humerus. Sutton & Nelson (1937) observed that high-zinc diets brought about anaemia, subnormal growth and reproductive failure in rats. This was confirmed by Smith & Larson (1946) who investigated the nature of the anaemia caused by zinc. According to them, it was microcytic and hypochromic, and the condition could be cured by additional supplements of copper while supplements of liver or liver extracts caused resumption of growth.

During the present investigation, it was observed that the high-zinc diets brought about a reduction in the weight and also a reduction in fat content of the liver. Zinc appeared to have a lipotropic effect. Development of the bones was also adversely affected. A preliminary report of this work has already appeared (Sadasivan, 1950).

EXPERIMENTAL

Albino rats of the Haffkine inbred strain were divided into groups of six, with an equal distribution between the sexes in each group. All the rats were fed a basal diet consisting of the following (parts by weight): wheat (*Triticum vulgare*), 58; gram (*Cicer arietinum*), 12; peas (*Pisum sativum*), 12; rice (*Oryza sativa*), 6; green leaves (*Amaranthus* sp.), 6; milk, 6. The constituents, excepting the green leaves and milk, were ground to a powder and well mixed. Weighed quantities of the powder were worked into a dough with the proportionate quantities of milk and green leaves, steamed for 30 min. and fed to the animals *ad lib.* On this diet, rats have been observed to thrive well. The supplements of Zn were made as pure grade ZnO (Merck) at levels of 0·5 and 1·0% in groups 2 and 3 respectively. The experiment extended over a period of 15 days only, since a longer period resulted in the death of rats in group 3. The food intake for each 3-day period was noted and growth measurements made at the end of each 3-day period, and, at the close of the experiment, the animals were killed by ether and liver, kidneys and femurs removed for assay. The fresh weight of liver and kidneys was noted after superficial drying with filter paper. The livers were minced and extracted with acetone in a Soxhlet extractor to determine the fat content. The results are presented in Table 1.

The femurs were cleaned of adhering flesh by rubbing with cloth, extracted with ether-ethanol mixture and dried to constant weight. They were ashed in a muffle furnace after preliminary heating with a gas burner and the ash weight noted. The ash was dissolved in pure dil. HCl, made up to a volume of 100 ml. and Ca and P determined in measured portions of the solution. Ca was determined by precipitating it as the oxalate and titrating it against standard permanganate solution. P was determined by the method of Fiske & Subbarow (1925) and the results are summarized in Table 2.

It will be observed from these tables that, on a stock diet, supplements of ZnO lowered the growth rate, the weight of liver and its fat content. The addition of ZnO to the diet at an 0·5% level did not affect the food intake, but the higher

Table 1. *Analysis of livers of rats fed on a stock diet supplemented with zinc*

(Figures in brackets represent standard deviation.)

Group	Diet	Initial wt. (g.)	Final wt. (g.)	Average daily food intake (g.)	Fresh wt. of liver (g.)	Wt. of liver (as % of final wt.)	Fat in liver (g.)	Fat in liver (as % of liver wt.)	Fresh wt. of kidney (g.)
1	Stock diet	96·0	138·0	21·4 (±0·59)	5·585 (±0·13)	4·050 (±0·10)	0·3844 (±0·010)	6·89 (±0·58)	1·230 (±0·03)
2	Stock diet +0·5% ZnO	102·0	139·5	21·7 (±0·81)	4·823 (±0·17)	3·485 (±0·18)	0·2935 (±0·016)	5·88 (±0·24)	1·195 (±0·04)
3	Stock diet +1·0% ZnO	103·0	116·0	19·0 (±0·64)	4·458 (±0·04)	3·865 (±0·17)	0·2707 (±0·030)	6·07 (±0·64)	1·160 (±0·014)

Table 2. *Analysis of femurs of rats fed a stock diet supplemented with zinc*

(Figures in brackets represent standard deviation.)

Group	Diet	Final body wt. (g.)	Dry wt. of femurs (g.)	Ash wt. (g.)	Ash (%)	Ca (mg.)	P (mg.)	Ca/P ratio
1	Stock diet	138·0	0·3875 (±0·009)	0·1990 (±0·0065)	51·35 (±0·94)	68·28 (±2·14)	40·35 (±0·80)	1·692 (±0·066)
2	Stock diet +0·5% ZnO	139·5	0·3248 (±0·028)	0·1553 (±0·0153)	47·52 (±1·27)	54·51 (±5·39)	33·30 (±2·84)	1·637 (±0·030)
3	Stock diet +1·0% ZnO	116·0	0·2608 (±0·011)	0·0998 (±0·0098)	38·09 (±2·65)	32·63 (±3·52)	25·05 (±1·91)	1·303 (±0·046)

Table 3. *The analysis of livers of rats fed on a high-fat, low-protein diet supplemented with zinc*

(Figures in brackets represent standard deviation.)

Group	Diet	Initial wt. (g.)	Final wt. (g.)	Average daily food intake (g.)	Fresh wt. of liver (g.)	Wt. of liver (as % of body wt.)	Fat in liver (g.)	Fat content of liver (%)
1	10% casein, 30% fat diet	50	63	7·7	5·26 (±0·43)	8·35 (±0·56)	1·2775 (±0·296)	24·29 (±3·90)
2	As group 1 +0·5% ZnO	47	53	6·3	4·04 (±0·16)	7·62 (±0·22)	0·5727 (±0·118)	14·18 (±2·27)
3	As group 1 +1·0% ZnO	45	47	5·1	3·03 (±0·16)	6·45 (±0·19)	0·3687 (±0·080)	12·17 (±1·96)
4	10% casein, 10% fat diet	53	76	9·6	6·08 (±0·27)	8·00 (±0·15)	1·0367 (±0·127)	17·05 (±1·60)
5	As group 4 +1·0% ZnO	48	47	6·9	2·78 (±0·23)	5·96 (±0·23)	0·1420 (±0·039)	5·11 (±1·24)

Table 4. *Analysis of bones of rats fed on a high-fat, low-protein diet supplemented with zinc*

(Figures in parentheses represent standard deviation.)

Group	Diet	Final body wt. (g.)	Dry wt. of femurs (g.)	Ash wt. (g.)	Ash (%)	Ca (mg.)	P (mg.)	Ca/P ratio
1	10% casein, 30% fat diet	63	0·2430 (±0·008)	0·0898 (±0·0075)	36·95 (±0·56)	34·8 (±0·79)	14·70 (±1·58)	2·53 (±0·31)
2	As group 1 +0·5% ZnO	53	0·2195 (±0·009)	0·0643 (±0·0027)	29·29 (±0·64)	24·4 (±1·21)	11·00 (±1·07)	2·22 (±0·20)
3	As group 1 +1·0% ZnO	47	0·1863 (±0·009)	0·0512 (±0·0022)	27·48 (±1·34)	18·6 (±0·84)	8·55 (±0·44)	2·21 (±0·16)
4	10% casein, 10% fat diet	76	0·2477 (±0·009)	0·1120 (±0·0028)	45·22 (±0·53)	40·8 (±0·99)	19·52 (±0·32)	2·09 (±0·04)
5	As group 4 +1·0% ZnO	47	0·1563 (±0·011)	0·0502 (±0·0055)	32·12 (±1·32)	17·6 (±2·30)	8·74 (±1·07)	2·01 (±0·04)

level of supplement markedly decreased the intake of food. The analysis of the femurs of rats showed that Zn appreciably reduced the dry weight and ash content of the bones. Evidently, the supplement interfered with development and mineralization.

The analysis of livers presented in Table 1 shows that Zn may act as a lipotropic agent though this fact is not unequivocally demonstrated by the results obtained on rats fed a stock ration. In order to confirm this point, Zn was added to a high-fat, low-protein diet on which rats normally developed fatty livers in about 15 days. Rats about a month old and of weight ranging between 40 and 60 g. were divided into five groups of six rats each. The first three groups were fed a diet consisting of ethanol-washed casein (choline content 1·5 mg./100 g.) 10 parts, hydrogenated groundnut oil 30, salt mixture (McCollum's) 4, sucrose 55 and shark-liver oil (12,000 i.u. vitamin A/g.) 1 part. In addition, the diet contained 15 μg. each of aneurin, riboflavin, and pyridoxine hydrochloride, 50 μg. of niacin and 20 μg. of calcium pantothenate/g. Groups 4 and 5 were fed a diet similar to the above but with only 10 instead of 30% fat, the difference being made up with sucrose. Groups 2, 3 and 5 received supplements of ZnO at 0·5, 1·0 and 1·0% levels, respectively. The low-fat groups (groups 4 and 5) were included in the series in order to find out the extent to which Zn proved toxic or lipotropic, as the case may be, when the fat content of the diet was low. The rats were weighed every 4th day and the food intake for each 3-day period was noted. At the end of the 15 days, the animals were killed and analysis of livers and femurs carried out as described earlier. These results are presented in Tables 3 and 4.

DISCUSSION

The results presented in this paper show that zinc in comparatively high concentrations causes a reduction in the weight and the fat content of rat livers. The reduction is marked in rats fed on a high-fat, low-protein diet and the results suggest that zinc possesses lipotropic activity.

Zinc brings about other changes also. On a stock diet, the 0·5% level of supplementary zinc oxide does not appear to lessen food intake, though it affects growth rate appreciably. On the stock ration, normal rats showed an increase in weight of 42 g. during an experimental period of 15 days. When, however, zinc oxide was added to the diet, the corresponding increases in weights were 37·5 and 13 g. for 0·5 and 1·0% levels of zinc oxide, respectively. The fresh weight of the kidneys also showed a corresponding decrease. At the 1% level zinc oxide caused a lowered food intake.

On a high-fat, low-protein diet, these changes were more pronounced. However, the decrease in the weight of the livers and their low fat content was particularly striking when the fat content of the diet was 10%. The content of fat in the diet thus appears to influence the changes brought about by the element.

Tables 2 and 4 show clearly that the development of bones is affected adversely in both the series. The observations of Smith & Larson (1946) that high-zinc diets bring about an anaemia, retarded growth rate and lowered food intake in rats, and the results presented in this paper show that zinc probably affects metabolism in general and assimilation of phosphorus in particular.

SUMMARY

1. The effect of supplements of zinc at levels of 0·5 and 1·0% on the livers and bones of rats has been investigated.

2. Zinc supplements caused a marked lowering of the fat content of livers of rats fed on a diet on which control rats developed fatty liver.

3. Zinc appears to act as a lipotropic factor in the concentrations studied. This element also interferes with development and mineralization of bones.

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In vitro Measurements of the Turnover Rate of Potassium in Brain and Retina

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It has been established by the isotope technique (Joseph, Cohn & Greenberg, 1939; Hahn, Hevesy & Rebbe, 1939; Fenn, Noonan, Mullins & Haege, 1941–2; Noonan, Fenn & Haege, 1941) that the potassium ions of animal tissues and of the body fluids continually interchange. The high concentration gradients of potassium between tissues and blood plasma, therefore, cannot be due to permeability barriers, but must be the resultant of two opposing processes: 'leakage' from the tissues, which tends to level out the concentration differences, and 'active', i.e. energy-fed, transport into the tissues, which counteracts the effects of 'leakage'. Under normal conditions the rates of the two opposing processes are usually equal and the problem presents itself of determining their velocities, i.e. the 'turnover rate' of potassium. Data published by Fenn *et al.* (1941–2) and Noonan *et al.* (1941) indicate great variations of the velocities from tissue to tissue. In general the visceral organs exchange potassium much more rapidly than skin, resting muscle or red blood cells. The only material for which accurate quanti-

tative data are available are human red blood cells. On the average 1·6–2·0 % of the cellular potassium exchanges in 1 hr., the agreement between the measurements of three independent teams being satisfactory (Raker, Taylor, Weller & Hastings, 1950; Sheppard & Martin, 1950; Solomon, 1950).

Terner, Eggleston & Krebs (1950) reported previously that slices of brain tissue and pieces of retina maintain their normal potassium content when kept in a saline medium containing oxygen, glucose and L-glutamate. If one of these substrates is absent the potassium concentrations in tissue and medium level out, but the loss of potassium to an inadequate medium can be restored by the addition of the missing substrate. Tissues suspended in saline media thus represent a system in which the exchange of ions with the medium can be studied. The present paper is concerned with measurements of the 'turnover rate' of potassium in brain cortex and in retina. ^{42}K was added to the saline medium and the rate of disappearance of the isotope was measured. From these measurements the turnover rate is calculated.

A preliminary account of this work was given to the Biochemical Society on 17 June 1950 (Krebs, Eggleston & Terner, 1950).

METHODS

Incubation of tissues. Guinea pig brain was handled as described by Stern, Eggleston, Hems & Krebs (1949). The technique of Deutsch (1936) was used for slicing brain cortex and the procedure of Terner *et al.* (1950) for preparing pieces of ox retina. The thickness of the brain slices was about 0·4 mm., that of the retina about 0·25 mm. Immediately after weighing on the torsion balance, the tissue, about 100 mg. fresh wt., was put into the medium in a conical Warburg vessel. The medium consisted of 2 ml. of bicarbonate saline supplemented by various substrates. The gas space was filled with 5 % CO_2 and 95 % O_2. All operations were carried out as quickly as possible and about 2 min. were required for preparing the tissue and the manometer for incubation. The manometers were attached to the water bath at 2 min. intervals, shaken at 40° and removed after varying periods of incubation.

The quantities of tissue and saline were so chosen that the initial K content of both was about equal. This favoured accuracy of the measurements.

Handling of ^{42}K. For reasons given later ^{42}K was not mixed with the medium at the start, but after a preliminary incubation period of 45 min. A solution of KCl (0·1 ml.) containing ^{42}K was placed in the side arm of the manometer flask, the quantity of ^{42}K being adjusted so as to give about 4000 counts/min. The amount of total K in the side arm was 0·008–0·032 mg., i.e. about 2–8 % of the K in the main compartment. Thus the additional amount of K did not appreciably change the level of K in the medium. At the end of the incubation period the tissue was removed from the medium, drained by rolling on a glass plate, weighed on a torsion balance and dropped into 2·5 ml. of 0·84 % NaCl solution. This was stored in the refrigerator for about 4 days to allow for the decay of ^{42}K. The tissue was then ground up in a mortar and 1 ml. of the suspension was diluted with 10 ml. of 0·84 % NaCl solution. This diluted tissue suspension was used for the determination of K by flame photometry according to Domingo & Klyne (1949).

The medium in the Warburg cup, after the removal of the tissue, was washed out with several portions of 0·1 N-KCl solution, and medium and washings were made up to a total volume of 10 ml. A KCl solution rather than water was used for washing to prevent retention of ^{42}K by the glass vessel (see Crane & Davies, 1949). The radioactivity of the solution was measured in the liquid Geiger-Müller counting tube of Veall (1948) (Type M-6, 20th Century Electronics Ltd). Corrections were made for isotope decay, dead time and background counts and also for the amount of medium removed from the cup with the tissues (see Terner *et al.* 1950). The error in determination of the radioactivity was of the order of ± 3 %.

The intial K content of the medium and of the tissue was measured on separate samples of the materials; the total K content of tissue plus medium was therefore known. The K content of the medium at the end of the incubation was calculated from the observed K content of the tissue and the total K content of the system. The reliability of this procedure was tested by direct determination of the K content of the medium in special experiments. The value was found to agree within the limits of error with the calculated value.

RESULTS

Changes in the potassium content of brain slices and retina on incubation in vitro

The quantitative analysis of data on the uptake of ^{42}K by tissues is much simplified if the concentration of total potassium ($^{39}K + ^{42}K$) in the tissue remains constant on incubation. Preliminary tests were therefore carried out to examine whether this holds true. In the previous experiments (Terner *et al.* 1950) the potassium concentration of brain slices found after 40–60 min. incubation was, in fact, of the same order as the initial concentration, but further experiments show that the tissue potassium concentration does not remain constant throughout the period of incubation (Table 1). A substantial loss of potassium occurs during the first 5 min. This is largely recovered within the following 20–30 min. and subsequently the level remains approximately constant for 20–30 min. In some experiments the stabilized potassium level was about the same as the starting value, in others it was up to 20 % below the starting value. With ox retina, as already described previously, potassium is lost during transport from the abattoir. During the initial stages of incubation the potassium of the tissue rises to a level similar to that found in fresh tissue and remains constant for the 30–60 min. period. The main experiments with ^{42}K therefore began after a preliminary incubation period of 45 min., when the potassium concentration in brain and retina had become approximately stabilized. The use of homologous blood serum did not prevent the initial loss of potassium by the tissue

Table 1. *Changes in the concentration of potassium in guinea pig-brain cortex and ox retina on incubation* in vitro

(Each vessel contained 2 ml. medium and 80–100 mg. tissue (wet wt.). Glucose (0·02 M) and L-glutamate (0·01 M) were added to both saline and serum.)

Exp. no.	Tissue	Medium	Period of incubation (min.)	Amount of K found in tissue (m-equiv./kg.)			Amount of K found in medium (m-equiv./l.)	
				Before incubation	After incubation		Before incubation	After incubation
					Total	Change		
1	Brain cortex	Saline	5	99·0	52·9	−46·1	5·20	6·96
			10	99·0	69·0	−30·0	5·20	6·50
			20	99·0	76·0	−23·0	5·20	6·17
			40	99·0	79·4	−19·6	5·20	5·85
2	Brain cortex	Guinea pig serum	5	93·6	57·5	−36·1	7·32	9·04
			10	93·6	61·7	−31·9	7·32	8·97
			20	93·6	74·2	−19·4	7·32	7·95
			40	93·6	73·5	−20·1	7·32	8·15
3	Brain cortex	Saline	30	98·4	77·5	−20·9	5·28	5·81
			40	98·4	94·5	− 3·9	5·28	5·28
			50	98·4	88·8	− 9·6	5·28	5·62
			60	98·4	82·9	−15·5	5·28	5·73
4	Brain cortex	Guinea pig serum	30	106·0	96·4	− 9·6	7·46	8·03
			40	106·0	98·4	− 7·6	7·46	7·70
			50	106·0	98·6	− 7·4	7·46	7·72
			60	106·0	86·2	−19·8	7·46	8·36
5	Brain cortex	Ox serum	30	101·5	33·4	−68·1	6·50	9·06
			40	101·5	30·9	−70·6	6·50	9·35
			50	101·5	28·2	−73·3	6·50	9·70
			60	101·5	24·7	−76·8	6·50	9·60
6	Retina	Saline	30	39·8	65·6	+25·8	5·32	3·68
			40	39·8	73·7	+33·9	5·32	3·51
			50	39·8	76·7	+36·9	5·32	3·04
			60	39·8	72·8	+33·0	5·32	3·03
7	Retina	Ox serum	30	40·2	59·8	+19·6	6·46	5·58
			40	40·2	67·6	+27·4	6·46	4·95
			50	40·2	71·6	+31·4	6·46	4·65
			60	40·2	82·2	+42·0	6·46	4·20

(Table 1); saline medium was therefore used in the main experiments. In heterologous serum brain slices lost more potassium than controls suspended in saline and the loss was not reversed on prolonged incubation.

Uptake of ^{42}K *by tissues.* A series of parallel manometer vessels containing the tissue suspended in the saline medium was incubated as described, and at graded intervals vessels were removed from the bath for analysis. One sample of tissue was put aside for the determination of the initial potassium content. Preliminary experiments showed that a measurable uptake of ^{42}K occurred within a few minutes and intervals of 2–4 min. were therefore chosen. The results are recorded in Table 2. The tenth column of the table shows that the radioactivity of the medium rapidly decreased whilst the total potassium content of the medium (eighth column) showed no major changes. The data confirm that a rapid exchange of potassium between tissue and medium occurs, the rate being somewhat greater in retina than in brain.

Calculation of the rate of potassium exchange. The following quantitative treatment of the data is based on three simplifying assumptions, namely: (i) That the total potassium content (i.e. $^{39}K + ^{42}K$) in tissue and in medium remains constant; in other words that the quantities of potassium ions moving into, and out of, the tissue per unit time are equal. The data in Table 2, columns 4 and 6, show that this supposition is approximately correct. (ii) That all tissue potassium is 'free'; i.e. that there is only one form of potassium in the tissue. That this assumption is approximately correct for brain and retina is borne out by the observation that 80–90 % of the tissue potassium rapidly diffuse into the medium when the energy supply is cut off by the withdrawal of oxygen and substrate. (iii) That the rate of potassium exchange under the experimental conditions (i.e. in thin layers of tissue) is a function of the whole tissue and not of the surface layer only. The basis for this assumption is the fact that the maintenance of the steady state depends on the supply of energy which

Table 2. *Migration of* ^{42}K *into brain slices and retina*

(Tissue suspended in bicarbonate saline containing 0·02 M-glucose and 0·01 M-L-glutamate. ^{42}K added as ^{42}KCl after 45 min. preliminary incubation.)

Exp. no.	Tissue: Organ	Tissue: Fresh weight (mg.)	Period of incubation after addition of ^{42}KCl (min.)	Amount of K found: Tissue: Before incubation (m-equiv./kg.)	Amount of K found: Tissue: After incubation (m-equiv./kg.)	Amount of K found: Medium: Before incubation (m-equiv./l.)	Amount of K found: Medium: After incubation (m-equiv./l.)	Radioactivity of medium (counts/min.): Before incubation	Radioactivity of medium (counts/min.): After incubation	Rate of exchange: (m-equiv. K exchanged/min.) ($\times 10^{-4}$)	Rate of exchange: m-equiv. K exchanged/min. / total tissue K $\times 100$: Individual slice	Rate of exchange: m-equiv. K exchanged/min. / total tissue K $\times 100$: Average
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
1	Brain	84	2	100·0	102·9	5·65	5·53	3470	3275	3·40	4·0	
		84	4	100·0	95·8	5·65	5·81	3470	3130	3·31	4·1	4·0
		77	6	100·0	89·0	5·65	6·06	3470	3050	2·96	4·3	
		84	8	100·0	91·0	5·65	6·03	3470	2960	2·80	3·7	
2	Brain	103	4	101·0	95·0	5·95	6·25	880	795	3·50	3·6	
		83	8	101·0	81·5	5·95	6·75	880	800	1·91	2·8	3·4
		80	12	101·0	91·0	5·95	6·35	880	725	2·64	3·6	
		97	16	101·0	96·1	5·95	6·18	880	640	3·31	3·6	
3	Brain	80	4	102·5	96·5	5·65	5·88	3410	3060	3·45	4·5	
		87	6	102·5	86·6	5·65	6·33	3410	3005	3·03	4·0	4·5
		90	8	102·5	96·0	5·65	5·94	3410	2700	4·20	4·9	
4	Brain	86	8	102·5	107·1	5·95	5·75	3275	2690	3·30	3·6	
		105	12	102·5	93·8	5·95	6·41	3275	2550	3·28	3·3	3·5
		88	16	102·5	100·9	5·95	6·03	3275	2380	3·28	3·7	
5	Retina	127	2	58·5	63·2	5·95	5·65	3140	2930	4·21	5·3	
		125	4	58·5	76·7	5·95	4·82	3140	2510	6·14	6·4	7·4
		102	6	58·5	86·0	5·95	4·55	3140	2240	6·60	7·5	
		93	8	58·5	87·1	5·95	4·63	3140	1980	8·49	10·5	
6	Retina	109	2	68·1	73·1	5·95	5·68	4090	3570	8·54	10·7	
		132	4	68·1	73·5	5·95	5·60	4090	3160	8·69	9·0	10·7
		119	6	68·1	64·0	5·95	6·19	4090	2890	11·46	15·0	
		86	8	68·1	93·9	5·95	4·84	4090	2785	6·62	8·2	

is produced evenly throughout the tissues. It is difficult to assess the extent to which this assumption is valid. It is obvious that in a very thick piece of tissue loss of constituents from the surface layer must be more rapid than from the central layers. The critical thickness below which central and surface layers do not behave differently remains to be ascertained. Let a = quantity of total potassium in medium, b = quantity of total potassium in tissue, x = quantity of ^{42}K in medium at time t, y = quantity of ^{42}K in tissue at time t, v = quantity of total potassium passing from tissue to medium, and in the opposite direction, per unit time. A known amount (x_0) of ^{42}K is added to the medium at $t=0$. The total quantity of ^{42}K in the whole system is assumed to remain constant

$$x+y=x_0.$$

In the time dt the quantity of ^{42}K passing into the tissue is

$$\frac{x}{a}v\,dt,$$

and the quantity of ^{42}K travelling in the opposite direction is

$$\frac{y}{b}v\,dt.$$

Therefore $$\frac{dx}{dt}=v\left(\frac{y}{b}-\frac{x}{a}\right)=v\left(\frac{x_0-x}{b}-\frac{x}{a}\right).$$

Integrating

$$x=x_0\frac{a}{a+b}+A\exp\left[-vt\left(\frac{1}{a}+\frac{1}{b}\right)\right].$$

For $t=0$, $x=x_0$, so that

$$A=\frac{x_0 b}{a+b}.$$

Hence $$x=\frac{ax_0}{a+b}+\frac{bx_0}{a+b}\exp\left[-vt\left(\frac{1}{a}+\frac{1}{b}\right)\right]$$

or $$v=\frac{ab}{t(a+b)}\ln\frac{bx_0}{x(a+b)-ax_0}. \qquad (1)$$

This equation may be used to calculate the rate of potassium exchange (v) from sets of data for t and x. It is noteworthy that the value of v is independent of the units in which x and x_0 are expressed. The absolute values of x and x_0 are therefore not required for the calculation of v, the relative values, as measured by the Geiger counter, being sufficient. The turnover rate, expressed as percentage of tissue potassium turned over per unit time equals $v/a \times 100$. The accuracy of the evaluation of v is evidently low when the value of x is either near x_0 or near its maximum, $ax_0/(a+b)$; x should be between 30 and 70 % of its maximum.

The problem of calculating the turnover rate in the type of system under consideration has been treated before by Cohn & Brues (1945), Sheppard (1948), Raker *et al.* (1950) and Sheppard & Martin (1950). The present treatment is similar to that of the previous workers, especially that of Sheppard & Martin. It is somewhat simpler and more suited to the particular experimental data. It should be noted that the theory is independent of, and supplies no information on, the mechanism by which the ion exchange is effected.

On applying equation (1) to the measurements recorded in Table 2 the figures given in the last two columns of this table are obtained. For the purpose of the calculation it is ignored as unimportant that the potassium concentration in the tissue and in the medium were for practical reasons measured in different units (mg./kg. and mg./l. respectively). The figures for the turnover rates can be regarded as reasonably consistent, considering that each figure is based on a considerable number of independent measurements, which are all liable to error, and that the premises on which the theory rests are only approximately true. The data for brain show less scattering than those for retina, although the handling of retina, which did not involve slicing, might be expected to be less injurious. Possibly differences in the histological structure of the various parts of the retina are responsible for some of the variations.

The average turnover rates for brain were between 3·5 and 4·0 % and of retina 7 and 10 %/min. Compared with the turnover rate of red blood cells (0·03 %/min. according to Raker *et al.* 1950; Sheppard & Martin, 1950; and Solomon, 1950) these rates are very high. In brain potassium exchanges on the average about 120 times and in retina about 250 times more rapidly than in human red cells.

Experiments on other tissues

The potassium levels of slices of guinea pig liver, pigeon pancreas and pigeon gizzard (smooth muscle) on incubation in saline media and serum were investigated in the same way as with brain and retina, but it proved impossible to maintain the normal potassium level of the tissue *in vitro*. Slices of these tissues are thus not satisfactory material for turnover rate studies. Guinea pig-kidney cortex, on the other hand, was found to behave similarly to brain.

Liver. Guinea pig-liver slices, containing initially 85–90 m-equiv. potassium/kg., lost 40–60 % of their potassium content on aerobic incubation in saline media or guinea pig serum. The loss occurred within the first 10 min. of incubation and was not prevented by the following additions to the saline media (0·01 M, unless otherwise stated): glucose (0·02 M), glucose plus L-glutamate, pyruvate, succinate, fumarate, citrate, α-ketoglutarate, oxaloacetate, adenosine triphosphate (0·002 M) in combination with various substrates. Addition of hormones known to play a role in the distribution of electrolytes, namely adrenaline (10^{-3}–10^{-7} M), ephedrine (10^{-3}–10^{-5} M) plus adrenaline, extract of adrenal cortex ('eschatin', Parke Davis and Co., 1–10 dog units/ml.) was like-

Table 3. *Effect of various substrates on the loss of potassium by slices of guinea pig-kidney cortex*

(About 100 mg. slices (wet wt.) incubated in 2 ml. bicarbonate saline; 40°; 5% CO_2 in O_2.)

			Amount of K in tissue		
				After incubation	
Exp. no.	Substrate (final concentration)	Period of incubation (min.)	Initially (m-equiv./kg.)	Found (m-equiv./kg.)	Change (%)
1	None	5	70·2	44·4	−27
	Glucose (0·02 M); L-glutamate (0·01 M)	5	70·2	54·0	−23
	None	10	70·2	44·2	−27
	Glucose (0·02 M); L-glutamate (0·01 M)	10	70·2	60·0	−15
	None	20	70·2	49·7	−29
	Glucose (0·02 M); L-glutamate (0·01 M)	20	70·2	64·5	− 8
	None	40	70·2	45·6	−35
	Glucose (0·02 M); L-glutamate (0·01 M)	40	70·2	51·8	−26
2	None	30	72·5	51·6	−29
	Glucose (0·02 M)	30	72·5	46·2	−36
	L-Glutamate (0·01 M)	30	72·5	61·3	−15
	Glucose (0·02 M); L-glutamate (0·01 M)	30	72·5	61·3	−15
3	None	30	66·3	44·0	−34
	Fumarate (0·01 M)	30	66·3	44·5	−33
	α-Ketoglutarate (0·01 M)	30	66·3	68·3	+ 3
	DL-Aspartate (0·02 M)	30	66·3	55·2	−17
	L-Glutamate (0·01 M)	30	66·3	53·2	−19
4	None	30	73·4	40·3	−45
	α-Ketoglutarate (0·01 M)	30	73·4	77·7	+ 6
	NH_4Cl (0·01 M)	30	73·4	26·3	−64
	NH_4Cl (0·01 M); α-ketoglutarate (0·01 M)	30	73·4	45·7	−38
	Citrate (0·01 M)	30	73·4	36·0	−51
5	None	30	75·5	51·5	−32
	Pyruvate (0·01 M)	30	75·5	47·6	−37
	Succinate (0·01 M)	30	75·5	51·6	−32
	Citrate (0·01 M)	30	75·5	34·2	−55
6	None	30	76·2	51·6	−32
	α-Ketoglutarate (0·01 M)	30	76·2	72·3	− 5
	α-Ketoglutarate (0·005 M)	30	76·2	63·7	−17
	α-Ketoglutarate (0·0025 M)	30	76·2	56·7	−26
	α-Ketoglutarate (0·00125 M)	30	76·2	52·3	−31

wise ineffective, as was an increased potassium concentration up to five times the normal level, i.e. 25 m-equiv./l. Anaerobically the potassium loss was of the order of 70%. After the initial loss the potassium level in the tissue became stabilized at concentrations still four to ten times higher than that of the medium. Similar results were obtained with pigeon liver.

Pancreas. The initial potassium concentration of pigeon pancreas was found to be 100–120 m-equiv./kg. On incubation in saline media 20–30% of the tissue potassium were lost, irrespective of the presence of substrates. Apart from glucose and L-glutamate and the intermediates of the tricarboxylic cycle, glutamine, asparagine, casein hydrolysate and α-ketoglutarate plus ammonium chloride were tested.

Pigeon gizzard. The muscular layer was examined as an example of smooth muscle. The initial potassium concentration was 115–120 m-equiv./kg., 50–80% of which were lost on incubation. Again addition of substrates was ineffective.

Kidney cortex. Data on the changes of the tissue potassium level of sliced guinea pig-kidney cortex on incubation in saline media are shown in Table 3. When no substrate was added about one-third of the tissue potassium was lost on aerobic incubation and about two-thirds on anaerobic. Addition of L-glutamate, L-aspartate and α-ketoglutarate to the medium reduced the potassium loss, the latter being the most effective substrate. At a concentration of 0·01 M, α-ketoglutarate always prevented the loss of potassium, whilst pyruvate, succinate, citrate, fumarate and glucose had no effect. As in brain cortex and retina there was a rapid initial loss even in the presence of α-ketoglutarate or glutamate. This was reversed on further incubation.

DISCUSSION

Turnover rates in vitro *and* in vivo. The question may be raised whether measurements of turnover rates on isolated tissues reflect the behaviour of the tissue *in situ*. No definite answer can be given, but we see no decisive arguments against the assumption

that slices of brain and pieces of retina maintain the potassium gradients between tissue and environment in the same way as the intact organ, and that the turnover rates found *in vitro* are of the same order as those *in vivo*. However, the behaviour *in vivo* remains to be tested by independent methods of investigation. The rapid and irreversible loss of potassium by some tissues, e.g. liver, is no doubt an indication of tissue damage, but even *in vivo* losses of potassium may readily occur as an early, perhaps the earliest, sign of damage, as a result of injury (Cuthbertson, 1936), of surgical operations (Wilkinson, Billing, Nagy & Stewart, 1950; Blixenkrone-Møller, 1949), of asphyxia (Cattell & Civin, 1938) or shock (Holmes, 1947).

Mechanism of potassium discharge by tissues. The rapid initial loss of potassium from freshly cut tissue slices bears on the mechanism by which potassium is discharged by the tissue. In two experiments on brain cortex recorded in Table 1, 47 and 39 % of the tissue potassium were lost within 5 min. The average rate of loss was thus 9 and 8 %/min. Other experiments, not recorded in the table, showed that most of the loss found after 5 min. occurred within the first 3 min. and that the rate of loss during the first minute was over 20 % of the tissue potassium. This is a much higher rate than the turnover rate of potassium in the steady state and it follows that the 'leakage' rate of potassium is not constant, the discharge being less in the steady state than in freshly prepared slices. It cannot be assumed, therefore, that the migration of potassium from the tissue to the medium is solely due to passive leakage. Actively controlling factors must also be operative, a conclusion which has already been drawn by other workers from observations on the increased rate of discharge following the stimulation of muscle or nerve.

The time course of the loss of and recovery of potassium by brain tissue recalls the rapid onset and gradual disappearance of the injury potentials of animal tissues. Changes in the tissue potassium concentration have long been assumed to be the main cause of these potentials (see Cowan, 1934).

Effect of heterologous serum. The fact that in heterologous serum brain tissue fails to maintain normal potassium levels may be ascribed either to an increased rate of potassium discharge or to a reduced rate of potassium uptake. As the respiration, and thus the energy supply, is not adversely affected by serum, it is more likely that the point of attack of serum is the mechanism controlling the discharge rather than the uptake of potassium. The loss of tissue potassium caused by heterologous serum may possibly have the same basis as the losses due to injuries (Cuthbertson, 1936), surgical operations (Wilkinson *et al.* 1950; Blixenkrone-Møller, 1949) or the action of hormones.

Potassium and glycogen synthesis. Buchanan, Hastings & Nesbett (1949) have found that the synthesis of glycogen from glucose and pyruvate in liver slices is optimal if the potassium concentration of the medium approximates to that of the tissues. This observation may be explained on the basis of the present results. A high potassium concentration in the tissue appears to be essential for the synthesis of glycogen. The synthesis is therefore bound to be adversely affected by the inability of liver slices to maintain their normal potassium level in the usual media. In rat diaphragm, which on incubation in a saline medium does not lose potassium ions (Kamminga, Willebrands, Groen & Blickman, 1950), an increased potassium concentration of the medium does not stimulate the rate of glycogen synthesis (Stadie & Zapp, 1947).

Energy relations. If the rate of exchange of potassium between tissue and environment is known, it is possible to calculate the energy required to maintain the concentration gradient using the formula

$$-\Delta F = \boldsymbol{R}T \ln \frac{C_1}{C_2},$$

where $-\Delta F$ is the change of free energy, or work of concentration, required to bring 1 g.mol. of potassium from the concentration C_1 to the concentration C_2, $\boldsymbol{R}$ the gas constant (1·987 cal./degree) and T the absolute temperature (see Borsook & Winegarden, 1931). In brain cortex C_1/C_2 is about 18 and 1 kg. of tissue contains about 0·1 g.mol. of potassium. The energy required for potassium transport by brain cortex is therefore 365 cal./hr./kg. Assuming the Q_{O_2} value of -18 µl./mg. dry wt./hr. (Krebs, 1950) and wet wt./dry wt. ratio for guinea pig-brain cortex of 6 (based on measurements in this laboratory; see also Palladin & Bjeljaewa, 1924) the energy available for respiration is about 15,000 cal./hr./kg. Thus about 2·5 % of the available energy is required for the maintenance of the normal potassium level. Considering that potassium is only one of many diffusible cell constituents and that there may be concentration differences within the cells (see Opie, 1948, 1949), it would seem that a substantial proportion of the energy provided by brain respiration serves to maintain the unstable environment of the tissue.

SUMMARY

1. Slices of guinea pig-brain cortex lose about 40 % of their potassium content by discharge into the medium within a few minutes on being suspended in a saline medium or in homologous serum. The normal potassium content is restored in about 30 min. on aerobic incubation if glucose and L-glutamate are present; thereafter it remains approximately constant for 20–30 min.

2. After a preliminary incubation period of 45 min., i.e. after a steady state had been established, ^{42}K in the form of ^{42}KCl was added to slices of guinea pig-brain cortex and to pieces of ox retina, and the rate of migration of ^{42}K into the tissue was measured. From these measurements the rate of potassium exchange in the steady state was calculated. The turnover rate $\left(\frac{\text{potassium exchanged/min.}}{\text{potassium present in tissue}} \times 100\right)$, was 3·5–4 in brain cortex and 7–10 in retina.

3. With liver (guinea pig, pigeon), pancreas (pigeon) and smooth muscle (pigeon gizzard) the normal potassium gradient between tissue and environment was not maintained when the tissues were suspended in saline media or homologous serum. The bearing of this observation on the finding of Buchanan *et al.* (1949) that the rate of synthesis of glycogen in liver slices is optimal when the potassium concentration of the medium resembles that of the tissue is discussed.

4. The potassium level of sliced guinea pig-kidney cortex behaved similarly to that of brain and retina except that α-ketoglutarate was more effective in maintaining the normal level than L-glutamate.

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Studies in Detoxication

35. SPECTROPHOTOMETRIC DETERMINATION OF β-GLUCURONIDASE USING *p*-CHLOROPHENYLGLUCURONIDE AS SUBSTRATE

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In recent years, there has been a considerable interest in the enzyme β-glucuronidase. Its only proved activity is the hydrolysis of conjugated β-glucuronides, but the work of Levvy and his co-workers (Levvy, Kerr & Campbell, 1948; Karuhairatnam, Kerr & Levvy, 1949*a*; Kerr, Campbell & Levvy, 1949, 1950; see also Odell & Fishman, 1950) shows that when there is an increase in proliferative activity in certain tissues there is also an increase in the β-glucuronidase activity. The enzyme has been found to occur in increased amounts in cancer tissue (Fishman & Anlyan, 1947; Karunairatnam, Kerr & Levvy, 1949*b*; Odell & Burt, 1949) and in tissues undergoing repair after damage (Levvy *et al.* 1948). The use of β-glucuronidase as an agent for the smooth hydrolysis of β-glucuronides is likely to increase,

because hydrolysis by acid often leads to low yields of the aglycone. There is little doubt that several β-glucuronidases occur in nature, for glucuronidase activity has been detected in a variety of animal tissues, in plants (e.g. the baicalinase of Miwa (1932, 1934–5, 1936–7) appears to be a β-glucuronidase), in bacteria (Buehler, Katzman, Doisy & Doisy, 1949; Barber, Brooksbank & Haslewood, 1948) and in moulds (Spencer & Williams, 1950*a*).

The assay of β-glucuronidase can be carried out by a determination of either the glucuronic acid or the aglycone set free during its hydrolytic action on conjugated glucuronide. Most earlier methods depended on the non-specific reducing properties of glucuronic acid, but more recent methods rely on the estimation of the freed aglycone, and the monoglucuronides of oestriol, phenolphthalein and phenol have been used for this purpose. The use of oestriol monoglucuronide (Fishman, 1939) as a substrate is limited by its relative inaccessibility. Phenylglucuronide, however, is easily obtained (Garton, Robinson & Williams, 1949), but the estimation of phenol by the Folin-Ciocalteu reagent is subject to variable blanks due to protein (Kerr, Graham & Levvy, 1948). Phenolphthalein monoglucuronide has not been obtained crystalline and is only available as a cinchonidine salt after a somewhat tedious isolation procedure (Di Somma, 1940; Talalay, Fishman & Huggins, 1946; Fishman & Talalay, 1947; Fishman, Springer & Brunetti, 1948). Nevertheless, the use of phenolphthalein glucuronide appears to us to be one of the best methods for the assay of β-glucuronidase.

The present method arose as a result of two observations, the one being the ease with which pure crystalline *p*-chlorophenylglucuronide could be prepared and the other, the sharp differences in ultraviolet absorption spectra between the anion and the glucuronide of *p*-chlorophenol. The principle involved in the present determination of *p*-chlorophenol in the presence of its glucuronide is a general one and is applicable to the determination of a number of hydrolytic enzymes where the substrate, whose absorption spectrum is practically the same in acid and alkaline solution, contains a phenol whose spectrum in alkali is different from that in acid. The same principle is involved in the phenolphthalein glucuronide method for the assay of β-glucuronidase, but in this case the colour measured is in the visible part of the spectrum. A method of the same type is already being applied in this laboratory to sulphatase determinations using *p*-chloro- and *p*-nitro-phenylsulphuric acids as substrates (Robinson, Spencer & Williams, 1951).

EXPERIMENTAL

Preparation of p-*chlorophenylglucuronide.* The preparation of this compound, described by Spencer & Williams (1950*b*), has been improved as follows. A total of 6 g. of *p*-chlorophenol (m.p. 42·5°) was fed as a warm water emulsion to four rabbits. The 12 hr. urine (250 ml.) was filtered through glass wool, made 0·2 N with respect to HCl and then continuously extracted with ether for 16 hr. The glucuronide crystallized as plates in the extract, and after cooling to 0° was filtered off. Concentration of the filtrate yielded further crystals. The combined solids (6·7 g.) were recrystallized twice from small volumes of hot water with the addition of charcoal. The monohydrate of the glucuronide was thus obtained pure as long colourless plates (yield 5·2 g.), m.p. 151° and $[\alpha]_D^{21°}$ −82° (*c*, 0·5 in water).

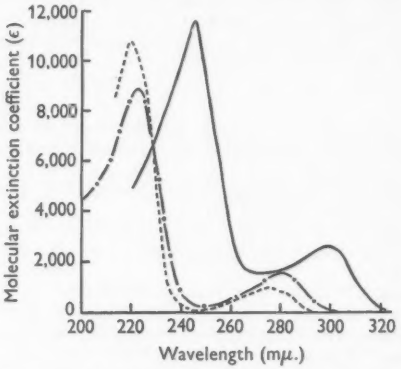


Fig. 1. Ultraviolet absorption spectra of *p*-chlorophenol in 0·1 N-NaOH (——) and in 0·1 N-HCl (–·–·–), and of *p*-chlorophenylglucuronide in 0·1 N-NaOH (----).

Table 1. *Absorption spectra of* p-*chlorophenol and its glucuronide*

	p-Chlorophenylglucuronide monohydrate		*p*-Chlorophenol	
Solvent	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ.)	$\epsilon_{max.}$
Water	219·5	10,700	—	—
	275·5	930	—	—
0·1 N-HCl	220	10,700	222	8,900
	275·5	910	280	1,600
0·1 N-NaOH	219·5	10,800	245	11,650
	276	970	298	2,600

Absorption spectra. All absorption spectra were determined with a Unicam spectrophotometer, Model S.P. 500. The ultraviolet absorption spectra of *p*-chlorophenylglucuronide in 0·1 N-HCl and 0·1 N-NaOH are identical (Spencer & Williams, 1950*b*), but *p*-chlorophenol itself exhibits different spectra in these solvents (see Smith, Spencer & Williams, 1950). The relevant data have been redetermined and are given in Table 1 and Fig. 1. It is clear from these data that the absorption maxima of *p*-chlorophenol in 0·1 N-NaOH occur at wavelengths where the absorption of the glucuronide is minimal. Thus by measuring the increase of absorption in 0·1 N-NaOH at 245 or 298 mμ. of a *p*-chlorophenylglucuronide solution under-

going hydrolysis, the amount of *p*-chlorophenol formed can readily be calculated. It can be assumed for practical purposes that all the change in absorption at these wavelengths is due to the formation of *p*-chlorophenol since the error introduced by neglecting the decrease in absorption due to hydrolysis of the glucuronide is very small (see Table 2).

Table 2. *Molecular extinctions of* p-*chlorophenol and its glucuronide at* 245 *and* 298 *mμ. in* 0·1 N-NaOH

(100.*B*/*A* is the percentage error introduced by neglecting decrease in absorption due to hydrolysis of glucuronide.)

	Extinction (ε)		
λ (mμ.)	*p*-Chlorophenol *A*	*p*-Chlorophenylglucuronide *B*	100.*B*/*A*
245	11,600	60	0·5
298	2,600	5	0·2

The data presented in Fig. 2 show that the maxima of *p*-chlorophenol in 0·1 N-NaOH obey the Lambert-Beer law. The effect of the acetate buffer and ethanol in the concentrations used in the glucuronidase assay were also investigated. It was found that these substances had no effect on the extinction coefficients of the phenol when due allowance for them was made in the blank cell of the spectrophotometer.

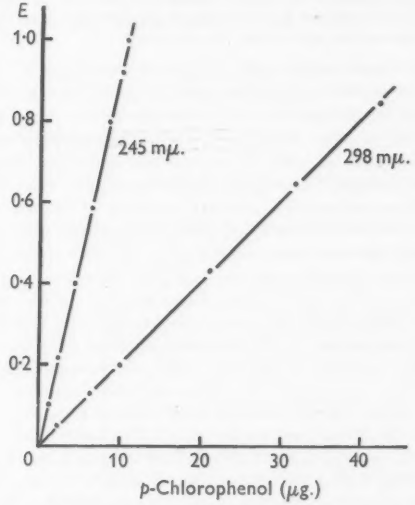


Fig. 2. Relation between the extinctions (*E*) at 245 and 298 mμ. and the concentration of the *p*-chlorophenol anion, demonstrating that the Lambert-Beer Law is obeyed.

Preparation of the glucuronidase extract. The extract used was obtained from ox spleen by a method based on the work of Talalay *et al.*(1946), Graham (1946) and Mills (1948). All pH measurements were made with the Cambridge pH-meter.

A freshly removed ox spleen was stripped of fat and the tissue (800 g.) homogenized with chilled water in an Atomix homogenizer. The temperature did not rise above 15°. The homogenate was centrifuged and the aqueous portion adjusted to pH 5 with N-acetic acid, and 25 ml. M-acetate buffer (pH 5) added to stabilize the pH. The mixture was then incubated for 1 hr. at 38°. The flocculated proteins were then centrifuged and the dark-red supernatant fluid (500 ml.) was half saturated with $(NH_4)_2SO_4$ and kept overnight at 0°. The precipitate which formed was collected on the centrifuge, dissolved in 200 ml. water and dialysed against running water for 24 hr. Further purification was effected by the method of Mills (1948) by precipitation with $(NH_4)_2SO_4$ between the limits of 1·25–1·54 M and 1·54–1·75 M, whereby two fractions (I and II) were obtained. Each fraction was twice reprecipitated between its limits and was stored at 0°. No deterioration in activity was observed during 3 weeks at this temperature.

Estimation of glucuronidase. For the glucuronidase estimations a mixture of the enzyme extract, substrate and buffer was incubated. The reaction was then stopped at the appropriate time and the liberated phenol estimated spectrophotometrically.

The most suitable buffer for the active pH range of the enzyme is 0·2 M-acetate buffer because its light absorption in the regions of the spectrum involved here is very small, and it does not inhibit the enzyme (Mills & Paul, 1949). The protein present in the enzyme extracts, however, has an appreciable absorption in alkaline solution at 245 and 298 mμ. and must be removed before the absorption due to *p*-chlorophenol can be measured. It was found that the protein could be completely precipitated and the reaction stopped by the addition of absolute ethanol to a final concentration of 80% (v/v). After removal of the protein by centrifugation, a portion of the solution was made 0·1 N to NaOH and the absorption measured in the spectrophotometer. No hydrolysis of the glucuronide solution occurred on incubation for 1–3 hr. with buffers at pH's between 5·6 and 3·75 and hence no blank of 'substrate incubated with buffer' was used in the estimation. Phenolic glucuronides as a rule required strong acids for hydrolysis and are stable to alkali. The final procedure adopted was as follows:

A series of four 15 ml. centrifuge tubes were set up. To two of these tubes (Tests) there were added, in order, 0·4 ml. each of 0·2-M-acetate buffer, *p*-chlorophenylglucuronide solution and enzyme extract. The solutions were mixed by rotating. All three solutions had been adjusted to the required pH and temperature of incubation before mixing. To the other two tubes (Controls) there were added 0·4 ml. each of 0·2-M-acetate buffer and enzyme extract adjusted to the required pH and temperature. All four tubes were then incubated at 37°. After the desired period of incubation, 0·4 ml. of the adjusted glucuronide solution was added to each of the control tubes. Then 4·8 ml. of absolute ethanol was added to each of the four tubes to stop the reaction and precipitate protein. The tubes were centrifuged for 3 min. to remove the protein. Then 5 ml. of the clear supernatant fluid were transferred from each tube to 10 ml. standard flasks, and made up to the mark with 0·2 N-NaOH. The extinctions of these solutions at 245 mμ. (1 mm. slit) were read in the spectrophotometer against a blank solution prepared as in the case of the test solutions except that 0·8 ml. of water had been used instead of the 0·4 ml. of the glucuronide solution and 0·4 ml. enzyme extract. In those instances where the β-glucuronidase activity was not known

approximately, and proved to be so great as to bring the extinction reading beyond the accurate portion of the spectrophotometer scale, accurate readings could be obtained by measuring the absorption at 298 mμ. (with 0·5 mm. slit) instead of at 245 mμ. Generally, however, with very active solutions of β-glucuronidase, estimations should be repeated by diluting the enzyme solution so that between 10 and 40 μg. of *p*-chlorophenol were liberated.

For readings taken at 245 mμ., the amount of *p*-chlorophenol liberated is given by the following equation:

$$\mu g.\ p\text{-chlorophenol} = \frac{(E_t - E_c) \times 128{\cdot}5 \times 6 \times 10{,}000}{11{,}650 \times 5} = 132{\cdot}3(E_t - E_c),$$

where, E_t and E_c are the observed extinctions of the test and control solutions respectively; 128·5 = mol. wt. of *p*-chlorophenol; 11,650 = molecular extinction of the *p*-chlorophenol anion at 245 mμ.

For 4 μg. *p*-chlorophenol, the value of $E_t - E_c$ is 0·03 which is easily read on the spectrophotometer.

The recovery of p-*chlorophenol.* Since the procedure described above involves the precipitation of protein, it was important to show that *p*-chlorophenol was not lost by adsorption on the protein. The recovery of added *p*-chlorophenol was tested by carrying out the procedure described for the test solutions in the preceding section, but replacing the glucuronide substrate by standard solutions of *p*-chlorophenol. The recovery of the phenol was tested with and without incubation and proved to be 100±4% for the ranges 8–425 μg. (Table 3). No chlorophenol was lost on the protein precipitate. Further experiments showed that small amounts of added *p*-chlorophenol could be recovered within ±1% in the presence of relatively large amounts of the substrate.

Table 3. *Recovery of* p-*chlorophenol in glucuronidase assay method*

(Method see text, p. 539; spectrophotometric determination of anion at 245 and 298 mμ.)

	p-Chlorophenol	
	Recovered	
Added (μg.)	Without incubation (%)	With incubation (%)
	Measured at 245 mμ.	
8·42	103	103·5
16·84	102·5	99
25·26	98	103
33·68	101·5	96·5
42·10	100	98
58·94	102	100·5
	Measured at 298 mμ.	
106·3	97	99
212·6	100	98·5
318·9	102	100·5
425·2	102·5	102

The effect of pH *on the activity of the enzyme*

The effect of pH on the β-glucuronidase activity of the fractions I and II in 0·2 M-acetate buffer was investigated using a substrate concentration of 0·015 M and incubating at 37° for 1 hr. The *p*-chlorophenylglucuronide solution was brought to the desired pH electrometrically by titration with 0·2 N-NaOH. The pH's of all solutions were checked with the Cambridge pH-meter. The pH-activity curves of fractions I and II were practically identical and the curve for fraction II is given in Fig. 3. Two maxima

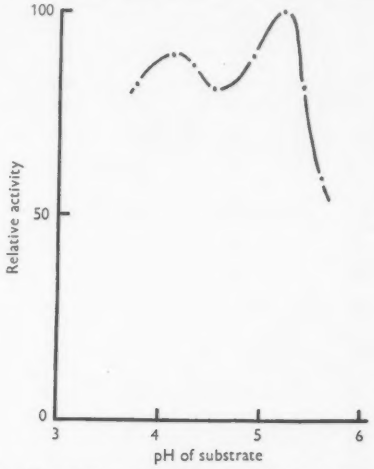


Fig. 3. pH-activity curve of fraction II (see text, p. 539) of an ox-spleen β-glucuronidase preparation. Activity expressed as percentage of maximum.

were found, one at pH 4·1 and the other at 5·2, the extracts showing greatest activity at the latter pH. Mills (1948) has reported the separation from ox spleen of two fractions showing β-glucuronidase activity with pH optima at 4·5 and 5·2, using *laevo*-menthyl, phenyl and phenolphthalein glucuronides as substrates. Further work by Mills and his co-workers (Mills & Paul, 1949; Mills, Paul & Smith, 1949) has demonstrated the presence of ox spleen of a third glucuronidase with a pH optimum of 3·4 acting only upon phenyl-β-glucuronide. Since the work reported in the present paper was done, reports have appeared in the literature (Sarkar & Sumner, 1950; Bernfeld & Fishman, 1950) which suggest that there is only one glucuronidase with an optimum pH at about 4·5. Although a pure enzyme preparation has not been used in this work the Lineweaver & Burk analysis below suggests that we may have been dealing with a single enzyme. It should be emphasized, however, that we are more concerned in this paper with the application of the spectrophotometric method than with enzyme kinetics.

The effect of substrate concentration and calculation of the Michaelis constant

The effect of varying the substrate concentration on the glucuronidase activity of fraction II at pH 5·2 and at 37° using an incubation period of

1 hr. is shown in Fig. 4. Optimal rate ($V_{opt.}$) was observed when the *p*-chlorophenylglucuronide concentration was $2{\cdot}85 \times 10^{-2}$M ($S_{opt.}$) and further increases in substrate concentration caused marked inhibition of the enzyme. Inhibition of glucuronidase activity by excess substrate has been previously observed by Talalay *et al.* (1946) with phenolphthalein glucuronide and by Kerr *et al.* (1948) with

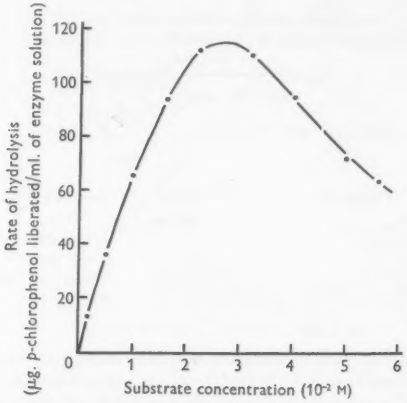


Fig. 4. Relation between substrate (*p*-chlorophenylglucuronide) concentration and β-glucuronidase activity.

phenylglucuronide, but neither group of workers analysed their data with a view to ascertaining the inhibitory mechanism involved. We have, however, been able to apply the tests proposed by Lineweaver & Burk (1934) to our data and have concluded that the mechanism involved is a pure substrate inhibition.

The reactions involved in substrate inhibition are

$$E + S \rightleftarrows ES \quad \text{and} \quad ES + (n-1)S \rightleftarrows ES_n,$$

where E = enzyme, S = substrate, ES is the active and ES_n is the inactive enzyme-substrate complex. The equation for general substrate inhibition derived by Lineweaver & Burk (1934) on the Michaelis-Menten treatment is

$$(S)/v = (K_m + (S) + (S)^n/K_2)/V_{max.}, \qquad (1)$$

where (S) = substrate concentration; v = observed velocity; K_m = dissociation constant of the ES complex, i.e. the Michaelis constant; K_2 = dissociation constant of ES_n complex; $V_{max.}$ = ideal maximal velocity of the enzyme reaction if there were no inhibition by excess substrate; n = number of molecules of substrate combined with enzyme-substrate complex.

The plot of [S]/v against [S] gave a concave curve which was linear at low values of [S] (Fig. 5). From the ratio of the ordinate intercept ($K_m/V_{max.}$) and the slope ($1/V_{max.}$) of the linear portion of the curve where substrate inhibition is negligible, the Michaelis constant K_m was calculated to be $3{\cdot}0 \times 10^{-2}$M.

The values derived from Fig. 5 were then used to plot $\log([S]/v - K_m/V_{max.} - [S]/V_{max.})$ against log [S], the graph of which is given in Fig. 6. From this figure the value of n, which is the slope drawn to the nearest whole number (Lineweaver & Burk, 1934), can be determined. In fact we found $n = 3{\cdot}95$ and, making $n = 4$, the value of K_2, the dissociation constant of the inactive complex, was calculated to be $6{\cdot}7 \times 10^{-5}$M.

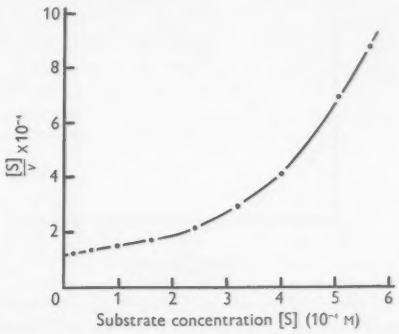


Fig. 5. Plot of substrate concentration [S] against [S]/v; see text, p. 541. (v is expressed in μg. *p*-chlorophenol liberated/ml. of enzyme solution.)

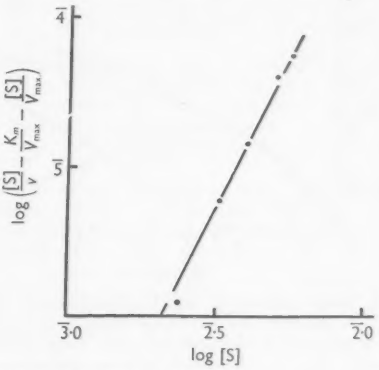


Fig. 6. Plot of log [S] against
$\log([S]/v - K_m/V_{max.} - [S]/V_{max.})$.
From the slope the value of n is calculated (see text, p. 541).

If the mechanism proposed is the true one, i.e. it is case III of Lineweaver & Burk (1934) then [S]/v plotted against $([S] + [S]^n/K_2)$ should give a straight line whose slope is $1/V_{max.}$ and ordinate intercept is $K_m/V_{max.}$. Fig. 7 shows that this plot is a straight line and furnishes good evidence that the proposed reactions are the ones operating. The data did not

conform with any of the other six cases analysed by Lineweaver & Burk. It will be shown later that sodium glucuronate is an inhibitor of glucuronidase, but in the concentrations liberated in this experiment its effect is negligible.

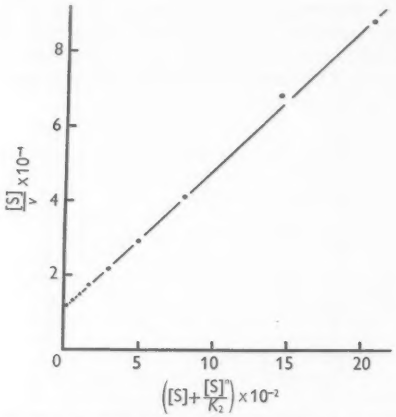


Fig. 7. Plot of $[S]/v$ against $([S]+[S]^n/K_2)$. The straight line shows that the proposed mechanism of competitive substrate inhibition is the true one (see text, p. 541).

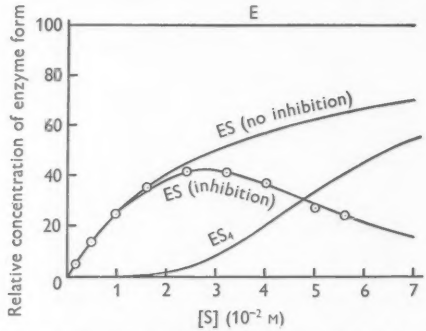


Fig. 8. Graph showing the relative distribution of E, ES (active) and ES_4 (inactive) as a function of substrate concentration. E represents the total concentration of enzyme free and combined; ES (no inhibition), the calculated concentration of active enzyme-substrate complex if there were no inhibition; ES_4, the calculated concentration of inactive enzyme-substrate complex; ES (inhibition) represents the calculated concentration of active enzyme-substrate complex when inhibition occurs. The points on the curve ES (inhibition) are experimental values (see Fig. 4).

From the values of K_m, K_2, and $V_{max.}$ obtained in the above analysis, the relative distribution of E, ES (active) and ES_n (inactive) has been calculated and is presented in Fig. 8. It will be noted that there is considerable agreement between the experimental results and the calculated curve for ES in the presence of ES_4.

The values of the various constants are given in Table 4.

Table 4. *Values of various constants for the action of β-glucuronidase on* p*-chlorophenylglucuronide at* pH 5·2

$V_{opt.}$ = optimum velocity; relative values taking $V_{max.} = 100$. The calculated value is obtained from the equation

$$V_{opt.} = \frac{(n-1)V_{max.}}{(n-1)+nK_m\sqrt[n]{\left[\frac{(n-1)}{K_mK_2}\right]}}.$$

The calculated value for [S] at $V_{opt.} = \{K_mK_2/(n-1)\}^{1/n}$.

Constant	Value: Calculated	Value: Observed (Fig. 4)
K_m	3×10^{-2} M	—
$K_{\frac{1}{2}V_{opt.}}$	—	$0{\cdot}82 \times 10^{-2}$ M
$V_{opt.}$	41·7	42
[S] at $V_{opt.}$	$2{\cdot}87 \times 10^{-2}$ M	$2{\cdot}85 \times 10^{-2}$ M
K_2	$6{\cdot}7 \times 10^{-5}$ M	—
n	4	—

Effect of time. Using extract II the effect of varying the incubation period on the release of *p*-chlorophenol by the enzyme was studied at pH 5·2 and 37° and a straight-line relationship was observed (Fig. 9) over a period of 3·5 hr. The maximum concentration of glucuronic acid liberated during this time was $2{\cdot}1 \times 10^{-4}$ M, an amount which is not inhibitory (see p. 544). Similarly, *p*-chlorophenol was not inhibitory in the concentrations liberated in these experiments.

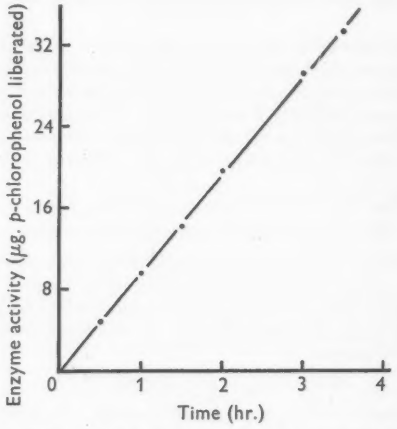


Fig. 9. Relationship of enzyme activity to time (0·4 ml. of suitably diluted extract II at pH 5·2 and 37°, with $1{\cdot}5 \times 10^{-2}$ M-*p*-chlorophenylglucuronide).

Effect of enzyme concentration. The results are shown in Fig. 10, which shows a straight-line relationship between the amount of enzyme and *p*-chlorophenol liberated up to 50 μg.

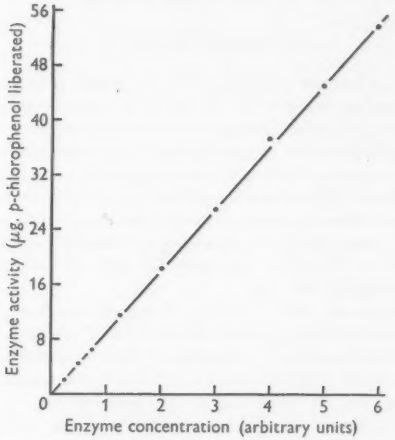


Fig. 10. Plot of enzyme activity (expressed in μg. *p*-chlorophenol liberated) against relative enzyme concentration (in arbitrary units). Conditions as for final assay procedure, p. 543. Extract II, pH 5·2, 37°; time of incubation, 1 hr.; substrate concn., $1{\cdot}5 \times 10^{-2}$M.

Final assay procedure

From the results of the above experiments it was concluded that optimal β-glucuronidase activity occurred at a substrate concentration of $2{\cdot}85 \times 10^{-2}$M after a 1 hr. incubation at 37° and pH 5·2. Various considerations, which include conservation of substrate, the value of control readings and activity, lead us to recommend a substrate concentration of $1{\cdot}5 \times 10^{-2}$M for general assay work. At this concentration, activity of the enzyme is 80% of the maximum value and the control reading, due to the absorption of the substrate which remains constant, is about 0·250, bringing the test readings into the most accurate portion of the spectrophotometer scale. For convenience and accuracy the enzyme solutions used should be diluted so that they liberate between 10 and 40 μg. *p*-chlorophenol.

The assay procedure is the same as detailed on p. 539, using $1{\cdot}5 \times 10^{-2}$M-substrate, all solutions being adjusted to pH 5·2 and 37° before mixing. An incubation period of 1 hr. is used. Under these conditions the amount of *p*-chlorophenol liberated is proportional to the enzyme present (see Figs. 9, 10).

Unit. A *p*-chlorophenyl unit of β-glucuronidase activity may be defined as the amount of enzyme which at pH 5·2 and 37° in $1{\cdot}5 \times 10^{-2}$M-*p*-chlorophenylglucuronide and 0·2M-acetate buffer liberates 1 μg. of *p*-chlorophenol in 1 hr.

The effect of inhibitors on β-glucuronidase

The inhibition of β-glucuronidase by various compounds has been investigated by Karunairatnam & Levvy (1949) who examined the inhibitory effects of certain sugar acids and a variety of miscellaneous substances. The most potent inhibitor was D-glucosaccharic acid. These results were largely confirmed by Mills & Paul (1949), who in a brief communication reported inhibition by some dibasic acids and monobasic and dibasic polyhydroxy acids which were not named in their communication. Our main interest was the application of the spectrophotometric method to the study of β-glucuronidase, but in view of the differences in structure between D-glucurone and D-glucuronic acid it was interesting to apply our method to the study of the inhibition of glucuronidase by these substances.

Materials

D-Glucurone, m.p. 171–173°, $[\alpha]_D^{22°} + 19{\cdot}85°$ (*c*, 1·76 in water) was the gift of Corn Products Refining Co.

Sodium D-glucuronate monohydrate was prepared by adding exactly one equivalent of 0·1N-NaOH to a solution of D-glucurone. On mixing the two substances, the mixture turned yellow, but the colour faded in 10 min. After keeping overnight the solution was concentrated to a small volume *in vacuo* and the Na salt precipitated by addition of ethanol. A repetition of the precipitation process gave the salt as a white amorphous powder with $[\alpha]_D^{22°} + 22{\cdot}5°$ (*c*, 0·8 in water). (Found: Na, 10·0; H_2O, 7·5. Calc. for $C_3H_9O_7Na.H_2O$, Na, 9·8; H_2O, 7·7%.) Ehrlich & Rehorst (1929) record $[\alpha]_D + 22{\cdot}5°$ for sodium D-glucuronate monohydrate after mutarotation.

Sodium mucate was prepared by addition of the equivalent of 0·1N-NaOH to mucic acid, m.p. 222° (decomp.).

Sodium potassium D-glucosaccharate was similarly prepared from acid potassium saccharate.

Methods

The determinations were carried out as described for the estimation of glucuronidase (p. 543), using 0·2 ml. of inhibitor solution and 0·2 ml. *p*-chlorophenylglucuronide solution instead of the 0·4 ml. of glucuronide solution in both the test and control tubes. For comparison of the rate in the absence of the inhibitor, 0·2 ml. water was substituted for the inhibitor solution. All solutions were previously adjusted to pH 5·2.

Calculation of K_I, the dissociation constant of enzyme-inhibitor complex

Competitive inhibition can be represented by the reaction

$$2E + S + I \rightleftarrows ES \text{ (active)} + EI \text{ (inactive)},$$

where E = enzyme, S = substrate and I = inhibitor. By the treatment of Michaelis & Menten (1913), Lineweaver & Burk (1934) derive the equation

$$\frac{1}{v} = \frac{1}{V_{max.}}\left(K_m + \frac{K_m[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V_{max.}}, \qquad (2)$$

where [I] is the concentration of inhibitor and K_I is the dissociation constant of the enzyme-inhibitor complex EI. Other symbols are as defined on p. 541.

Lineweaver & Burk utilize the above equation by plotting $1/v$ against $1/[S]$ and competitive inhibition is shown by an increase in (slope $\times V_{max.}$), K_I being calculated from the values of the slope and intercept. However, in the present study there is the added complication of inhibition by excess substrate. This difficulty can be avoided by working at substrate concentrations where substrate inhibition is negligible, but this restricts the range of concentrations to 0–0·01 M (see Fig. 8), where glucuronidase activity is low. We have therefore overcome the difficulty by fixing the substrate concentration at a position where glucuronidase activity is highest without substrate inhibition being of importance (0·01 M) and then varying the concentration of the inhibitor. To suit our requirements the above equation (2) can be rearranged in the form $y = mx + c$ as follows:

$$\left(\frac{V_{max.}}{v} - 1\right) = \frac{K_m}{K_I[S]}[I] + \frac{K_m}{[S]}. \qquad (3)$$

If the inhibition is competitive the plot of $(V_{max.}/v) - 1$ against [I] should produce a straight line whose ordinate intercept is $K_m/[S]$ and slope

Table 5. *Dissociation constants of β-glucuronidase-inhibitor complexes*

Inhibitory	K_I (10^{-3} M)	Affinity ($1/K_I$)
Sodium potassium D-glucosaccharate	0·88	1136
Sodium D-glucuronate	11·1	90
Sodium mucate	36·2	28
D-Glucurone	Non-inhibitory	—

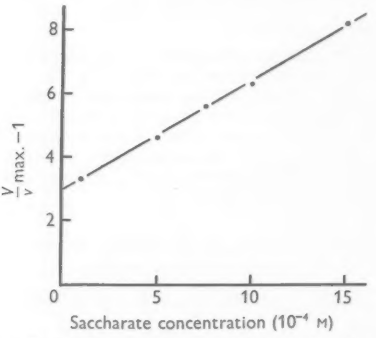


Fig. 11. Graph showing that the inhibition of β-glucuronidase by D-glucosaccharate is competitive (see text, p. 544).

$K_m/K_I[S]$, so that K_I is given by the ratio (intercept : slope). K_m and $V_{max.}$ for *p*-chlorophenylglucuronide are already known (see Table 4). The results of this method of analysis show that all the inhibitory compounds listed in Table 5 inhibit competitively, because, as shown for D-glucosaccharate in Fig. 11, the plot of $(V_{max.}/v) - 1$ against [I] is a straight line and the ordinate intercept is the calculated value of $K_m/[S] = 3$.

DISCUSSION

The procedure presented in this paper provides a convenient method for the assay of β-glucuronidase. Although no claim is made that the present procedure is superior to that using phenolphthalein glucuronide, it possesses considerable advantage in the case of preparation of the substrate, *p*-chlorophenylglucuronide. Using this compound, optimal β-glucuronidase activity was found at a substrate concentration of $2{\cdot}85 \times 10^{-2}$ M when measured at pH 5·2 and incubated at 37° for 1 hr.

Applying the spectrophotometric method, the kinetics of the enzymic reaction have been studied and the inhibition occurring at high substrate concentration has been shown, following the analytical procedure of Lineweaver & Burk (1934), to be probably due to the formation of an inactive enzyme-substrate compound of suggested formula ES_4. The analysis has also provided a value of the Michaelis-Menten constant ($K_m = 3 \times 10^{-2}$ M). The recorded Michaelis constants for β-glucuronidase acting on various substrates are given in Table 6.

Table 6. *Michaelis constants (K_m) of β-glucuronidase*

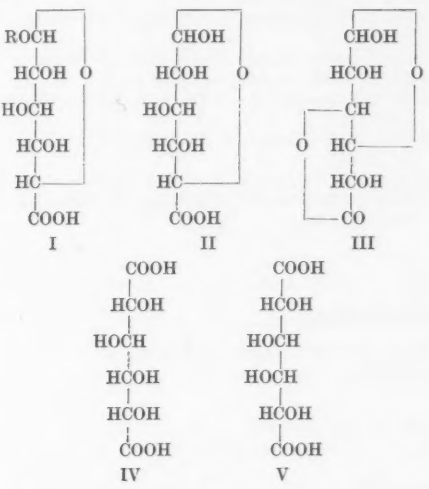
Substrate	K_m (M)	pH	Reference
Phenolphthalein monoglucuronide	0·00005	4·5	Talalay *et al.* (1946)
laevo-Menthyl-glucuronide	0·004	5·3	Fishman (1939)
Oestriol mono-glucuronide	0·005	4·3	Fishman (1939)
laevo-Bornyl-glucuronide	0·01	4·4	Fishman (1939)
Phenyl-glucuronide	0·0035*	5·2	Kerr *et al.* (1948)
p-Chlorophenyl-glucuronide	0·03	5·2	This paper

* $K_{\frac{1}{2}V_{opt.}}$ in this instance (see text).

All the values of K_m quoted in this table except that of phenylglucuronide have been calculated according to Lineweaver & Burk (1934). The substrate concentration-activity curve of phenylglucuronide does not lend itself to calculation of K_m by this method, and the figure quoted by Kerr *et al.* (1948) is actually $K_{\frac{1}{2}V_{opt.}}$. Since excess substrate inhibition by this glucuronide is strong, the value of $K_{\frac{1}{2}V_{opt.}}$ does not represent the true Michaelis-Menten constant.

The values of K_m and $V_{max.}$ (the true maximum velocity) calculated for *p*-chlorophenylglucuronide enabled us to determine the dissociation constants

(K_I) of the complexes of β-glucuronidase with certain sugar acid inhibitors. The literature contains only one previous value of K_I, that for D-glucosaccharic acid by Karunairatnam & Levvy (1949). Their value, 6×10^{-5}M, was only about 1/15 of our value of $8{\cdot}8 \times 10^{-4}$M. In their calculation of K_I from equation (3) when $V_{max.}/v = 2$, Karunairatnam & Levvy have actually used $K_{\frac{1}{2}V_{opt.}}$ for K_m and V_{opt}/v for $V_{max.}/v$.



Three sugar acids and one sugar acid lactone were tested for inhibitory action on β-glucuronidase (see Table 6). In agreement with Karunairatnam & Levvy (1949), D-glucosaccharic acid was found to be the most powerful inhibitor, its affinity for the enzyme being 13 times that of D-glucuronic acid and 40 times that of mucic acid. Karunairatnam & Levvy do not clearly distinguish in their paper between glucurone and glucuronic acid, but they have informed us, in a personal communication, that their method of making up the glucurone solution very probably converted it to glucuronic acid; it appears very probable that the inhibition they record for glucurone was due to glucuronic acid. We found that D-glucurone did not inhibit β-glucuronidase. On theoretical grounds it might be expected that the enzyme would be inhibited by glucuronic acid but not by glucurone, because these compounds have totally different structures, glucuronic acid (II) being a pyranose (Challinor, Haworth & Hirst, 1931; see also Smith, 1939), and glucurone (III) a furanose derivative (Smith, 1944).

The sodium D-glucuronate used by us was a mutarotated form containing the α- and β-isomers and we cannot say whether the inhibition is due to both forms or due to the β-form only (cf. I which is a β-glucuronide, a normal substrate for β-glucuronidase). Since D-glucosaccharic acid (IV) is a powerful inhibitor of the enzyme, it appears probable that a strong inhibitor should possess two carboxyl groups and chain of CHOH groups of similar configuration to that of the glucuronide (I). D-Glucurone has neither a carboxyl group nor the necessary hydroxyl groups on C atoms 3 and 4. Mucic acid (V) has two carboxyl groups, but not the necessary configuration of CHOH groups and is less inhibitory than glucuronic acid, which has one carboxyl plus the necessary configuration of CHOH groups.

SUMMARY

1. It has been shown that β-glucuronidase can be determined spectrophotometrically using *p*-chlorophenylglucuronide as substrate, since *p*-chlorophenol in alkali has absorption bands at 245 and 298 mμ. where the absorption of the glucuronide is negligible.

2. The method has been applied to an ox spleen glucuronidase preparation and the results examined by the analytical methods of Lineweaver & Burk (1934). The inhibition of the enzyme by excess substrate has been shown to be competitive.

3. Various constants for the action of β-glucuronidase on *p*-chlorophenylglucuronide have been calculated.

4. Inhibition of the enzyme by D-glucosaccharic, mucic and D-glucuronic acids and D-glucurone has been studied. Glucurone was found to be non-inhibitory and the powerful inhibitory action of glucosaccharic acid has been confirmed. These results have been discussed.

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Studies in Detoxication

36. A NOTE ON THE GLUCURONIDES OF BENZENEAZOPHENOL AND BENZENEAZORESORCINOL

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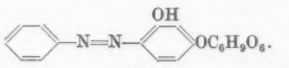
(*Received* 8 *July* 1950)

Salant & Bengis (1916) found that a number of fat-soluble dyes administered to rabbits were excreted in the urine and bile, besides being deposited in adipose tissues. Since no fat was excreted in the urine with these dyes, these workers concluded that they were converted in the body into water-soluble substances, probably ethereal sulphates and glucuronides. Benzeneazophenol (oil yellow) and benzeneazoresorcinol (Sudan G 35) were fed to rabbits and the urine was examined for metabolites. Glucuronides were isolated in both cases and although melting points were recorded, the compounds were not analysed or properly described.

It appeared from the paper of Salant & Bengis that the glucuronides of benzeneazophenol and benzeneazoresorcinol would be compounds readily prepared by acidification of urine, and we thought that they might perhaps be suitable substrates for the assay of β-glucuronidase, if the glucuronides were less highly coloured than the free azophenols. We have therefore prepared and characterized these glucuronides and examined their possibilities as substrates for glucuronidase.

We have assumed that the monoglucuronide obtained from benzeneazoresorcinol is 4-benzeneazo-3-hydroxyphenylglucuronide (I) on analogy with the biosynthetic monoglucuronides of 4-chlororesorcinol (Dodgson & Williams, 1949) and 2:4-dihydroxyacetophenone (Dodgson, 1950), which have been proved to be *para*-substituted phenyl glucuronides (see also Dodgson, Smith & Williams, 1950).



(I)

EXPERIMENTAL

4-Benzeneazophenyl-β-D-glucuronide. Benzeneazophenol (m.p. 151–152°; 2 g.) was fed to a rabbit (weight, 3 kg.). After 3 hr. the bright-yellow urine (50 ml.), which gave negative tests for aromatic amino groups and reducing substances but positive ones for glucuronic acid, was acidified with HCl. A small pale-yellow crystalline precipitate of the glucuronide separated; this, however, redissolved on further acidification.

To isolate the glucuronide, the urine was made faintly acid to litmus and extracted three times with a mixture of 1 vol. ethanol to 3 of ether. The extracts were evaporated to a syrup to which was added Na_2SO_4 to form a paste. The latter was then stirred repeatedly with small amounts of the ethanol-ether mixture until all the yellow material had been extracted. Evaporation of this solution gave

crystals of the glucuronide (200 mg.), which on recrystallization from methanol gave the 4-*benzeneazophenylglucuronide* as pale-yellow needles of the *sesquihydrate*, m.p. 164°, $[\alpha]_D^{20°} - 88°$ (*c*, 0·7 in ethanol). Salant & Bengis (1916) give m.p. 164–165°. (Found: C, 54·3; H, 5·4; N, 7·3. $C_{18}H_{18}O_7N_2.1{\cdot}5H_2O$ requires C, 54·1; H, 5·3; N, 7·0%.) It was slightly soluble in ethanol and ethyl acetate, less soluble in ether and almost insoluble in water.

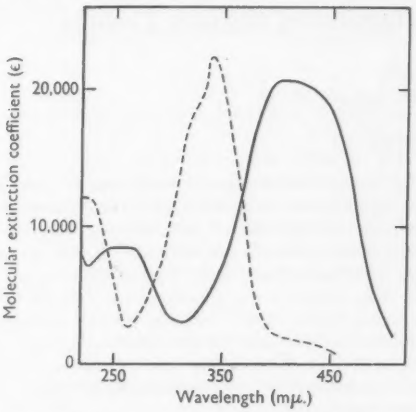


Fig. 1. Absorption spectra of benzeneazophenol (——) and its glucuronide (– – – –), in 0·1 N-NaOH. Benzeneazophenol: $\lambda_{max.}$ 250–270 mμ., $\epsilon_{max.}$ 8500 and $\lambda_{max.}$ 400–410 mμ., $\epsilon_{max.}$ 20,700. Benzeneazophenylglucuronide sesquihydrate: $\lambda_{max.}$ 336 mμ., $\epsilon_{max.}$ 22,400.

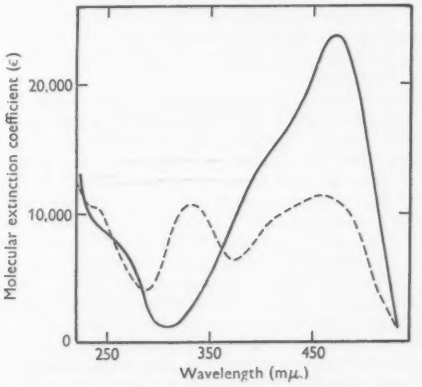


Fig. 2. Absorption spectra of benzeneazoresorcinol (——) and its monoglucuronide (– – – –), in 0·1 N-NaOH. Benzeneazoresorcinol: $\lambda_{max.}$ 470, $\epsilon_{max.}$ 24,000. Benzeneazoresorcinol monoglucuronide monohydrate: $\lambda \sim 230$ mμ., ϵ, 12,000; $\lambda_{max.}$ 333 mμ., $\epsilon_{max.}$ 11,100 and $\lambda_{max.}$ 460 mμ., $\epsilon_{max.}$ 11,700.

4(?)-*Benzeneazo*-3(?)-*hydroxyphenyl*-β-D-*glucuronide*. 4-Benzeneazoresorcinol (British Drug Houses Ltd., m.p. 161°; 2 g.) was fed to a rabbit (weight 3 kg.) and the urine (100 ml.) collected for 15 hr. Tests on the urine were similar to those for benzeneazophenol urine. The urine was made acid to congo red and kept at 0° for several hours. The crystalline deposit was filtered, washed with water on the centrifuge and recrystallized from ethanol-water. The 4(?)-*benzeneazo*-3(?)-*hydroxyphenylglucuronide monohydrate* was obtained as yellow needles (200 mg.), m.p. 198°, $[\alpha]_D^{20°} - 114°$ (*c*, 1 in ethanol). Salant & Bengis (1916) give m.p. 189–190°. Its solubilities were similar to those of the benzeneazophenol derivative. (Found: C, 53·0; H, 5·3; N, 7·1. $C_{18}H_{18}O_8N_2.H_2O$ requires C, 53·2; H, 5·0; N, 6·9%.)

Spectroscopic observations

Both the azophenols and their glucuronides formed yellow solutions which were not very obviously altered in colour when made alkaline. These glucuronides could not, therefore, be used as substrates for glucuronidase in the same way as phenolphthalein glucuronide, i.e. by the use of the red colour given in alkaline solution by phenolphthalein freed from the glucuronide by the enzyme. However, the absorption spectra of the azophenols and their glucuronides were examined in alkaline solution with the Unicam spectrophotometer (see Figs. 1 and 2).

DISCUSSION

The spectra of benzeneazophenol and its glucuronide (Fig. 1) in alkaline solution show considerable differences. Benzeneazophenol has a broad maximum at 400–410 mμ. with a high extinction ($\epsilon_{max.}$ 20,700), but its glucuronide also has an appreciable absorption at these wavelengths which amounts to almost 10% of that of the phenol, a fact which, we feel, renders the glucuronide not very suitable for glucuronidase assay. Benzeneazoresorcinol glucuronide is unsuitable for a similar reason (see Fig. 2); benzeneazoresorcinol has a sharp maximum at 470 mμ. ($\epsilon_{max.}$ 24,000), but the glucuronide at this wavelength has an extinction (ϵ) of about 11,000.

SUMMARY

1. The monoglucuronides of the fat-soluble dyes, benzeneazophenol and benzeneazoresorcinol have been prepared from the urine of rabbits receiving these dyes orally, and have been characterized.

2. The spectra of the glucuronides were compared with those of the parent dyes and it was concluded that the glucuronides were not suitable as substrates for β-glucuronidase assay.

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The Specificity of Phosphomonoesterases in Human Tissues

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Phosphates are present in many human and animal tissues, and the activity of these enzymes may easily be estimated, but it is very difficult to draw conclusions about their physiological role. In discussing the significance of the phosphatases, we must consider not only the enzyme activity but also the substrate; first, whether it has access to the enzyme and, secondly, how the enzyme acts upon it. This second question leads to the problem of the specificity of phosphatases.

The alkaline phosphatases of ossifying cartilage, kidney or intestine, and the acid phosphatases of prostate and red blood cells are not highly specific. King & Delory (1939), considering the relative rates of hydrolysis, pointed out that the rate depends on the acidity of the phosphoric ester, the more acid esters being more easily hydrolysed by the phosphatases.

Besides these non-specific phosphatases there exists a highly specific phosphatase which acts only on nucleotides with the phosphate group attached to the fifth carbon of the pentose, i.e. adenosine-5-monophosphoric acid (muscle adenylic acid; this nucleotide, for brevity, will be referred to as adenylic acid) and inosine-5-monophosphoric acid (inosinic acid). It does not act on the closely related yeast adenylic acid (adenosine-3-monophosphoric acid), or on adenosinetriphosphoric acid. This enzyme was first described in Parnas's laboratory by Reis (1934) in heart muscle and has been named 5-nucleotidase. Later it was found in nervous tissues (Reis, 1937*a*), and in many other tissues, especially lungs, testicles, foetal membranes, retina and choroid (Reis, 1937*b*, 1940). Animals may be classified as 'rich' or 'poor' in 5-nucleotidase; in the rat it is present in almost every tissue, while in the pigeon it was found only in the lungs. The presence of this enzyme in brain was confirmed by Fleischhacker (1938). Gulland & Jackson (1938) found it very active in snake venom, and Mann (1945) demonstrated a still greater activity in bull seminal plasma. Reis (1950) studied the distribution of 5-nucleotidase in human tissues and found the activity was highest in the thyroid, testicle and aorta walls. At the physiological pH in all human tissues, perhaps with the exception of prostate gland and intestinal mucosa, adenylic acid was much more easily hydrolysed than phenylphosphoric acid or β-glycerophosphoric acid.

The object of the present work was to compare the activities of 5-nucleotidase and non-specific phosphatases at different pH values. Estimations at a physiological pH are sufficient to demonstrate 5-nucleotidase and non-specific phosphatase activity, and may indicate the physiological role of these enzymes; but in order to indicate which non-specific phosphatase (the 'acid' or the 'alkaline') is present, estimations at alkaline and acid pH are necessary, and complete pH-activity curves may be traced with advantage.

METHODS

Since both the 5-nucleotidase and the non-specific phosphatase attack adenylic acid, the activity of 5-nucleotidase in a tissue extract can be deduced by measuring the activity against adenylic acid and subtracting that measured against some other phosphoric ester. For this purpose phenylphosphoric acid seems very suitable (King & Armstrong, 1934) as it has a higher rate of hydrolysis than many other phosphoric esters.

Tissue extracts. The tissues were usually taken 24 hr. after death. They were homogenized in a blender with distilled water in the proportion 1:20. The tissue homogenates, after the addition of $CHCl_3$ or thymol, were left for 2 days at room temperature for autolysis to occur. After that time the activity of the extracts was usually found to have increased, except in hot summer weather when it decreased, and hence it was thought better to keep the homogenates in a refrigerator. After 2 days the whole was centrifuged, and the supernatant liquid used for estimations of enzyme activity. The extracts kept in a refrigerator with thymol remained active for months. In order to diminish the phosphate content the extracts were dialysed in collodion tubes. This reduced the phosphate to about one-tenth of the original value without significant change in the activity of phosphatases.

Enzymic activities. These were tested by adding to 0·4 ml. of a buffer solution (0·05 M-biphthalate, 0·05 M-diethylbarbiturate, or Delory & King's (1945) carbonate-bicarbonate buffer, according to the desired pH) 0·1 ml. of 0·005 M substrate solution (adenylic acid, phenylphosphate or β-glycerophosphate, pH adjusted to 7·5). The mixture was placed in a water bath at 38°, and after 5 min. 0·1 ml. of the tissue extract, diluted if necessary, was added. After the desired incubation time, usually 0·5 hr., 1·0 ml. of 5% (w/v) trichloroacetic acid was added and the sample centrifuged. The clear supernatant was decanted into another tube and used for the phosphate estimation, which was based on the Fiske & Subbarow (1925) method. To develop the colour, 0·1 ml. of 10% ammonium molybdate in 30% (v/v) H_2SO_4, and 0·05 ml. of 0·2% aminonaphthol-

sulphonic acid in a solution of 12% (w/v) sodium metabisulphite and 2·4% sodium sulphite (crystalline), were added. In order to avoid inhibitory effects the dilution of tissue extracts and the incubation times were chosen so that not more than 50% of the substrate was hydrolysed.

RESULTS

The results are presented in Table 1. Comparing the activities of the enzymes near their respective optimal pH's (that is for 5-nucleotidase at pH 7·5, and for the non-specific phosphatases at pH 5·5 and 9·0), human tissues fall into groups showing the following types of phosphomonoesterase activity:

(1) *5-Nucleotidase nearly free from non-specific phosphatase*: thyroid, testicle, aorta and other arterial walls.

(2) *5-Nucleotidase much more active than non-specific phosphatases*: nervous tissues and posterior lobe of pituitary; the latter has the highest activity of 5-nucleotidase of all human tissues.

(3) *Both enzymes of an approximately equal activity*: liver, lung, kidney medulla, anterior lobe of pituitary.

(4) *Alkaline phosphatase much more active than 5-nucleotidase*: the highest alkaline phosphatase activities were found in ossifying cartilage, choroid plexus and choroid membrane (of the eye); the alkaline phosphatase activity in kidney cortex was comparatively very low.

(5) *Non-specific phosphatase, but no or very little 5-nucleotidase*: prostate, highly active acid phosphatase, which is still considerable at the physiological pH; intestinal mucosa (duodenum, jejunum), a comparatively low activity of alkaline phosphatase.

At the physiological pH, in all tissues examined, except prostate and intestinal mucosa, the activity of 5-nucleotidase was several times greater than that of the non-specific phosphatases.

A more detailed study of phosphomonoesterase activity in human tissues is demonstrated in Figs. 1–3. Fig. 1 shows the influence of pH on the 5-nucleotidase activity of aorta. The hydrolysis of phenylphosphoric acid (non-specific phosphatases) is almost negligible with this tissue, and therefore the hydrolysis of adenylic acid is due almost entirely to 5-nucleotidase. The optimal pH is 7·8, very near to

Table 1. *The activity of phosphomonoesterases in human tissues*

(The enzyme activity is expressed as μg. P hydrolysed/hr./mg. wet wt. tissue corresponding to volume of extract used.)

	Enzyme activity			
	Phenylphosphoric acid			Adenylic acid
Tissue	pH 5·5	pH 7·5	pH 9·0	pH 7·5
Group 1				
Thyroid	0·22	0·06	0·22	4·8
Thyroid	—	0·05	0·30	5·1
Testicle	0·30	0·10	0·40	3·8
Aorta wall (media)	0·09	<0·03	0·06	2·2
Aorta wall (media)	—	<0·03	—	2·9
Group 2				
Brain (cortex)	0·5	0·2	0·5	1·3
Nerve (optic)	0·3	<0·1	0·9	2·6
Retina	0·1	<0·1	1·0	2·2
Pituitary posterior lobe	0·6	0·6	7·0	21·2
Group 3				
Pituitary anterior lobe	0·7	0·2	3·4	4·2
Liver	0·3	0·1	1·3	0·9
Lung	0·4	0·2	2·0	1·2
Lung	—	0·5	4·6	2·1
Kidney medulla	0·9	0·3	1·9	1·2
Group 4				
Kidney cortex	1·9	0·7	4·1	1·5
Kidney cortex	0·9	0·3	2·1	0·9
Choroid plexus	0·1	0·6	14·0	3·6
Choroid plexus	—	0·6	13·0	2·8
Choroid membrane	0·2	0·3	8·3	1·8
Ossifying cartilage	0·2	0·9	21·0	4·3
Ossifying cartilage	—	1·6	56·0	3·2
Group 5				
Duodenal mucosa	0·1	0·2	2·6	0·2
Jejunal mucosa	0·4	0·4	3·7	0·6
Prostate	1030	395	0·5	80

the physiological pH. Similar hydrolysis curves were obtained for thyroid gland and for testicle.

In many tissues 5-nucleotidase is found together with non-specific phosphatases. Fig. 2 shows the action of an extract of human brain. In agreement with the work of Giri & Datta (1936), two optima

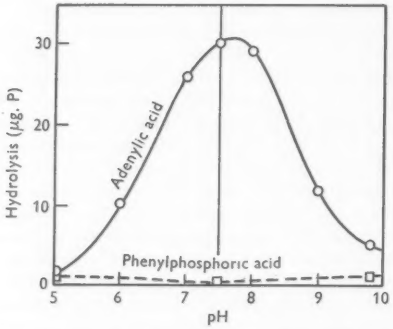

Fig. 1. Hydrolysis of adenylic acid and phenylphosphoric acid by human aorta wall. Dialysed extract corresponding to 25 mg. of tissue, 0·5 hr. at 38°.

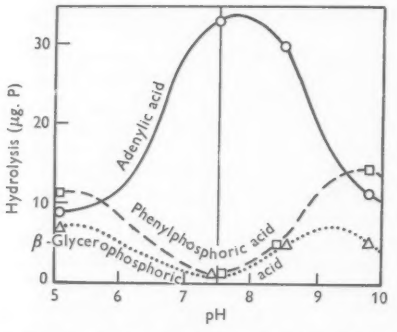

Fig. 2. Hydrolysis of adenylic acid, phenylphosphoric acid and β-glycerophosphoric acid by human brain. Dialysed extract corresponding to 25 mg. of tissue, 1 hr. at 38°.

were found for the non-specific phosphatases, an acid and an alkaline. These are demonstrated by the hydrolysis of phenylphosphate and β-glycerophosphate. As found by King & Delory (1939) the phenylphosphate is hydrolysed at a much greater rate than the β-glycerophosphate, and the pH optimum for phenylphosphate is more alkaline than that for β-glycerophosphate. The rate of hydrolysis of adenylic acid in acid and alkaline solutions is intermediate between the two other esters, but near the physiological pH it is very much greater. Adenylic acid is hydrolysed optimally near pH 7·5 at a point where the hydrolysis of phenylphosphate and β-glycerophosphate is at a minimum. Similar hydrolysis curves were found for human lungs and liver.

Fig. 3 represents the action of an extract of human ossifying cartilage. This tissue is the classical site of alkaline phosphatase. As in Fig. 2 the extent of hydrolysis of phenylphosphate is much greater than that of β-glycerophosphate; both curves show that the alkaline phosphatase action is very low at physiological pH, at pH 7·5 the hydrolysis of phenylphosphate being only about 5 % of the hydrolysis at

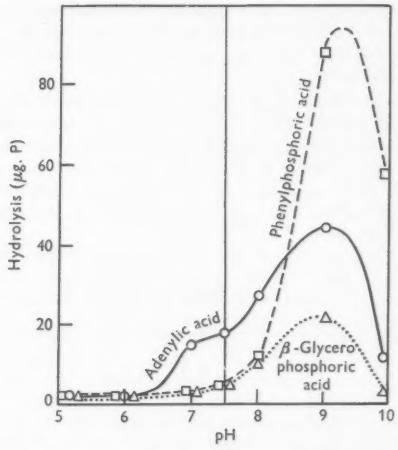

Fig. 3. Hydrolysis of adenylic acid, phenylphosphoric acid and β-glycerophosphoric acid by human ossifying cartilage. Extract corresponding to 8 mg. of tissue, 0·5 hr. at 38°. (Results at pH 9 and 10 for phenylphosphoric acid obtained from 15 min. hydrolysis, ×2.)

the optimal pH. At pH 7·5 the rate of hydrolysis of adenylic acid is about four times as great as the hydrolysis of the others esters. This indicates the presence of 5-nucleotidase, which, although comparatively weaker than the non-specific phosphatase at the optimal pH, is about four times more active than the latter at physiological pH. The action of extracts of kidney cortex and choroid plexus is similar to the above.

DISCUSSION

Newman, Feigin, Wolf & Kabat (1950) described the use of several phosphoric esters for a histological method of demonstrating phosphatase activity. They found that the hydrolysis of muscle adenylic acid in tissues is sometimes very different from that of the others phosphoric esters (e.g. in artery wall), and they rightly ascribed it to the presence of 5-nucleotidase. These authors failed to find any

5-nucleotidase activity in such human tissues as the thyroid gland, testicle or lungs. In the present experiments 5-nucleotidase was always very abundant in these tissues. The failure of Newman *et al.* may be explained by inactivation during histological fixation, and, which seems more important, by the fact that their work was carried out at pH 9·2, whereas 5-nucleotidase is optimally active near pH 7·8.

The physiological role of 5-nucleotidase is not known at present, but the existence of such an enzyme, specific for a substance so important and widely distributed as adenylic acid, seems to indicate a mechanism able to regulate the concentration of both phosphate and adenylic acid, which are so important for glycogenesis.

Since Robison's (1932) work it has been generally considered probable that the alkaline phosphatase plays a part in calcification of bones. As the 5-nucleotidase acts optimally near the physiological pH, where the alkaline phosphatase has a very low activity, it seems possible also that 5-nucleotidase may play a role in calcifications. It is of interest that certain tissues, e.g. aorta wall or thyroid gland, which are apt to undergo pathological calcifications, contain this enzyme, but not the alkaline phosphatase.

SUMMARY

1. At the physiological pH in all human tissues examined, with the exception of intestinal mucosa, the activity of the specific phosphatase, 5-nucleotidase, is much more pronounced than the activity of the non-specific alkaline phosphatase. This includes the tissues notably rich in alkaline phosphatase, e.g. ossifying cartilage, choroid plexus and kidney cortex.

2. The optimal pH for 5-nucleotidase from human tissues is pH 7·8.

3. The highest 5-nucleotidase activity in human tissues was found in the posterior lobe of the pituitary.

4. In adults the highest alkaline phosphatase activity (pH 9) was found in choroid plexus. The alkaline phosphatase activity of kidney cortex and intestinal mucosa was comparatively much lower.

5. The wide distribution of 5-nucleotidase seems to indicate a mechanism regulating phosphate and adenylic acid concentrations, which may perhaps play a part in the calcification of tissues.

I wish to express my thanks to Prof. E. J. King for his advice and the opportunity to perform the experiments in his laboratory.

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Observations on the Use of *Escherichia coli* for the Reduction and Estimation of Dehydroascorbic Acid

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Gunsalus & Hand (1941), working with suspensions of *Escherichia coli*, reported that the bacteria were capable of reducing dehydroascorbic acid to ascorbic acid in milk, wine and fruit juices, but seemed incapable of effecting the reduction with vegetable extracts. In these latter juices the *Esch. coli* acted as an oxidation catalyst and oxidized the ascorbic acid present.

Stewart & Sharp (1945), working with suspensions of *Esch. coli* and *Staphylococcus albus* and with a wide range of animal and plant extracts, were able to obtain a rapid and quantitative reduction of dehydroascorbic acid in such materials. Only after the complete removal of oxygen from these solutions, or under aerobic conditions if sodium cyanide was added, was there any reduction of dehydroascorbic

acid. They noted that, with the vegetable extracts in which the former workers had been unable to effect the reduction, complete reduction of dehydroascorbic acid to ascorbic acid occurred, provided that the ascorbic acid was extracted and titrated against indophenol dye at a pH not below 4·5. If the extract, prior to titration, was acidified to a pH of 3·5 or below, a rapid oxidation of ascorbic acid occurred. When, however, sodium cyanide was present in a concentration of 0·001 M the phenomenon did not occur and the dehydroascorbic acid was rapidly reduced to ascorbic acid which, under these conditions, could be titrated at pH values below 3·5 without oxidation. They attributed this behaviour to the fact that *Esch. coli* forms a compound or complex when incubated with vegetable juices which can oxidize ascorbic acid in acid solution below pH 3·5.

Being interested in alternative methods of estimating dehydroascorbic acid in plant extracts, we have re-examined the results of using bacterial suspensions for this purpose and have reported them below.

METHODS AND REAGENTS

Preparation of bacterial suspensions. Suspensions of *Esch. coli* were prepared by growing the organisms in Roux bottles on 2% digest agar containing 0·5% glucose for 18 hr. at 37%. The culture was harvested and washed twice with Ringer solutions diluted 1 in 4 and prepared from glass-distilled water. After the final washing, the culture was resuspended in the diluted Ringer solution to give a concentration of about 10^{10} or 10^{11} organisms/ml. Brown's opacity tubes were used for this standardization. No culture more than 18 hr. old was used since cultures older than this had lower enzyme activity. The activities of these suspensions were found to be constant over long periods, but, in practice, no suspension more than 7 days old was used in these experiments.

Bacterial suspensions, in which the viable organisms were destroyed by heat treatment at 60° for 1 hr., had lost the whole of their activity.

Estimation of ascorbic acid. Ascorbic acid was estimated by titration against standardized indophenol dye after acidification and arrest of the reaction by the addition of HPO_3 to a concentration of 4%.

Dehydroascorbic acid was prepared by dissolving a known amount of pure crystalline L-ascorbic acid in glass-distilled water, adding 1 drop of bromine, and removing the excess bromine rapidly by vigorous aeration. To avoid loss of dehydroascorbic acid this process was carried out as rapidly as possible and was completed within a period of 5 min. from the time of adding the bromine.

Buffers. For the estimation of ascorbic acid in biological material it is usual to extract the fresh tissue with metaphosphoric acid. In such an extract the ascorbic acid may be estimated by direct titration, and a further sample of the solution taken for estimation of total ascorbic acid (ascorbic acid + dehydroascorbic acid). For this reason in most of the experiments reported in this paper we have studied the reduction of dehydroascorbic acid by *Esch. coli* in a 2% (w/v) HPO_3 solution, buffered to pH 6·2 by the addition of NaOH. In some experiments a phosphate-citric acid buffer, 0·2 M, was employed. All buffer solutions were made up with glass-distilled water unless otherwise stated.

Temperature. These experiments were performed at 35°, like those of Stewart & Sharp (1945).

Amidosulphonic acid (NH_2SO_3H) solutions were freshly prepared from the pure dry substance.

Nitrite was estimated colorimetrically by means of the Griess-Ilosvay reagent, in a photoelectric colorimeter. Nitrate was detected qualitatively by means of the diphenylamine reagent.

Determination of the rate of the reaction. (*a*) *With pure dehydroascorbic acid.* To 50 ml. of buffer solution, 2 ml. of a glucose solution, 25% (w/v) was added. The reaction flask was placed in a water bath at a temperature of 35° and a rapid stream of N_2 passed. After 10 min., a suitable amount of bacterial suspension was added (in most experiments 4 ml.) and, finally, a measured amount of dehydroascorbic acid, ascorbic acid or other substance under test was added. The rate of reaction was followed by removing samples at suitable intervals. The reaction was arrested by the addition of 5 ml. of 5% (w/v) HPO_3 to 5 ml. of reaction mixture and the reducing titre of the solution determined by titration against standard indophenol dye. The pH of the solution was approx. 2·0. (*b*) *With potato extract.* The plant material was extracted in a Waring blender with 2% HPO_3, filtered and a 50 ml. portion neutralized to pH 6·2–6·3 with K_2HPO_4 40% (w/v). It was at once freed of O_2 by passing a rapid stream of N_2 through it for 5 min., the bacterial suspension was added, the reaction flask placed in the bath at 35° and the reaction followed by withdrawing samples at suitable intervals. The reaction was arrested in the usual way by the addition of HPO_3.

EXPERIMENTAL

Reduction of dehydroascorbic acid

We have confirmed the results of Stewart & Sharp (1945) that *Esch. coli* rapidly reduces dehydroascorbic acid under anaerobic conditions at pH 6·2 and at a temperature of 35°. Unlike them, however, we have not been able to obtain a quantitative recovery of added dehydroascorbic acid as ascorbic acid. We have found that the percentage of dehydroascorbic acid recovered depends on at least two factors: (1) the concentration of dehydroascorbic acid added, and (2) the enzymic activity of the bacterial suspension. This is illustrated in Figs. 1*a* and 1*b*. For Fig. 1*a* the concentration of dehydroascorbic acid was varied and the quantity of bacterial suspension kept constant, while for Fig. 1*b* the concentration of dehydroascorbic acid was constant and the enzymic activities were varied by altering the quantity of bacterial suspension added. The key to these results lies in the different periods of time taken for the reaction to be completed. Whether the time is prolonged by increasing the concentration of dehydroascorbic acid or by reducing that of the bacterial suspension, the percentage of dehydroascorbic acid recovered as ascorbic acid falls progressively. This is clearly illustrated in Fig. 2.

The explanation appears to be that dehydroascorbic acid is unstable and is converted to 2:3-diketogulonic acid by the opening of the lactone ring at a rate governed by pH and temperature. The reaction leads to irreversible destruction of dehydroascorbic acid. This explains that our failure to obtain a quantitative recovery of dehydroascorbic acid, as ascorbic acid, is because the rate of destruction of dehydroascorbic acid, as determined chemically, corresponds with the decrease in the percentage of dehydroascorbic acid recovered with bacteria in a series of tests in which the time taken for completing the reaction was varied (Fig. 2). In these experiments the disappearance of dehydroascorbic acid was followed chemically by determining the ascorbic acid regenerated after treatment with hydrogen sulphide, after suitable intervals of time, under the same conditions of pH and temperature as those used for the study of the reduction with the bacteria.

Penney & Zilva (1943), experimenting under very similar conditions, observed a conversion of approximately 39 % of dehydroascorbic acid to diketogulonic acid in 40 min., as compared with approximately 30 % found by us within the same time (Fig. 2). Since, under the conditions of our experiments, the rate of loss of dehydroascorbic acid over a period of 40 min. was approximately constant, it is possible to correct for it. The procedure would then be to follow the course of the reduction by the bacteria, determining the time taken for completion of the reaction, and then to apply a simple correction to give the total dehydroascorbic acid originally present. To reduce this correction to the minimum, highly active bacterial suspensions should be employed so as to give reaction times of short duration.

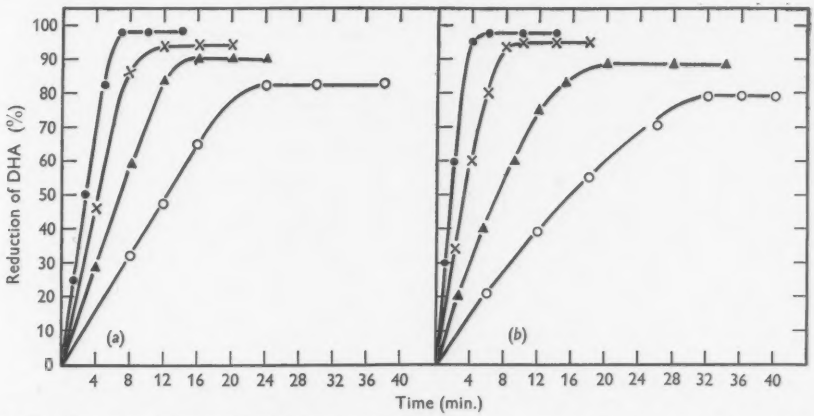

Fig. 1. (*a*) The effect of variation of dehydroascorbic acid (DHA) concentration on the percentage recovered as ascorbic acid (AA) using the same concentration of *Esch. coli*. ●—●, DHA 0·04 mg./ml.; ×—×, DHA 0·08 mg./ml.; ▲—▲, DHA 0·16 mg./ml.; ○—○, DHA 0·32 mg./ml. (*b*) The effect of altering the concentration of *Esch. coli* on the percentage DHA recovered as AA. ●—●, 4 ml. bacterial suspension in 50 ml. reaction mixture; ×—×, 2 ml. bacterial suspension in 50 ml. reaction mixture; ▲—▲, 1 ml. bacterial suspension in 50 ml. reaction mixture; ○—○, 0·5 ml. bacterial suspension in 50 ml. reaction mixture; DHA 0·08 mg./ml. reaction mixture.

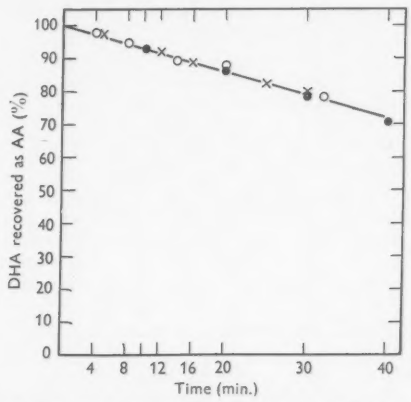

Fig. 2. The DHA (%) recovered as AA as affected by the time taken for the reaction to be completed compared with the rate of destruction of DHA determined chemically (H_2S reduction). ○—○, values obtained by variation of DHA concn.; ×—×, values obtained by variation of *Esch. coli* concn. (abscissae represent time in min. for the bacterial reaction to be completed); ●—●, disappearance of DHA as determined by reduction with H_2S.

The reason for the slight discrepancy between our results and those of Stewart & Sharp (1945), who reported quantitative recoveries of added dehydro-

ascorbic acid, is not clear. Gunsalus & Hand (1941) found that quantitative recovery was obtained only if the reaction was complete within 15 min. These latter workers prepared dehydroascorbic acid either by iodine oxidation or by enzymic oxidation with cucumber juice, whilst Stewart & Sharp prepared it by oxidation with cucumber extract. Gunsalus & Hand reported a better recovery with iodine than with ascorbic oxidase, and ascribed this to the fact that the longer time needed for the oxidation of ascorbic acid with the enzyme allowed more dehydroascorbic acid to be destroyed. It seemed possible that the difference in the method of preparing dehydroascorbic acid (we oxidized with bromine), might have been the cause of our failure to obtain quantitative recovery of dehydroascorbic acid. Experiments in which dehydroascorbic acid was prepared by oxidation with iodine, however, yielded similar results to those in which bromine was used; a result in accord with those of Penney & Zilva who found the rate of conversion of dehydroascorbic acid to diketogulonic acid to be the same whether iodine or chlorine was used as the oxidizing agent.

Neither of the groups of American workers give data showing the extent of the decrease in recovery of dehydroascrobic acid with longer reaction times, although both state that destruction of dehydroascorbic acid occurs under such circumstances. It is possible, however, that in their experiments the rate of destruction of dehydroascorbic acid was, for some unknown reason, slightly slower than in ours, and that the small percentage loss of dehydroascorbic acid that occurred in 15 min. was within the experimental error of their method of estimation.

Reduction of dienols other than ascorbic acid

Stewart & Sharp examined the reduction by *Esch. coli* of the oxidized forms of other non-specific reductants of indophenol dye. Glucose solutions previously treated with acid or alkali, sterilized evaporated milk, and canned fruit and vegetable juices were examined. No reduction of substances other than dehydroascorbic acid was observed in these products. They did, however, find that D-*iso*dehydroascorbic acid was reduced at a slower rate than L-dehydroascorbic acid.

We have determined the rate of reduction of a number of known dehydroenediol compounds. These included the oxidized forms of D-*iso*ascorbic acid, hydroxytetronic and reductic acids as well as reductone and reductone-like substances produced in an alkali-treated glucose solution.

The results with D-*iso*ascorbic acid, reductic and hydroxytetronic acids are shown in Fig. 3*a*. We found D-*iso*dehydroascorbic acid to be reduced at a slower rate than dehydroascorbic acid, 'though somewhat faster than the rate given by Stewart & Sharp. The oxidized forms of both hydroxytetronic and reductic acid were reduced much more slowly than dehydroascorbic acid. However, if these substances were present with ascorbic acid some interference would result. Thus, in the minimum time for complete reduction of dehydroascorbic acid the percentage reduction of an equivalent concentration of the oxidized forms of reductic and hydroxytetronic acids would be 28 and 9 respectively. The estimation of dehydroascorbic acid by means of *Esch. coli* must, therefore, be used with discretion if substances of this type are known to be present.

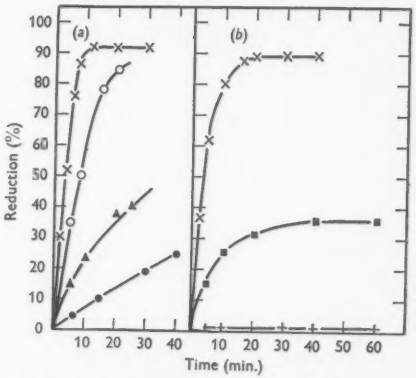

Fig. 3. The rate of reduction by *Esch. coli* of the oxidized forms of certain enediols compared with DHA. *a*, ×—×, L-ascorbic acid; ○—○, D-*iso*ascorbic acid; ▲—▲, reductic acid; ●—●, hydroxytetronic acid. *b*, ×—×, L-ascorbic acid; +—+, reductone (enoltartronaldehyde); ■—■, glucoreductone solution.

When pure reductone (enol of tartronaldehyde) was incubated over a period of 60 min. with *Esch. coli* no reduction of the oxidized form occurred.

Using a solution of glucoreductone prepared by treatment of glucose with alkali (Harris & Mapson, 1947) (this contains reductone and unidentified reducing substances closely akin to the indophenol reductants (cf. Mapson, 1943) produced in dried foods during processing or storage), some regeneration of the reducing power was effected by *Esch. coli* (Fig. 3*b*). Regeneration of 36% of the original indophenol titre occurred within 40 min., suggesting that one of the reductants in such a solution was reduced by *Esch. coli*, whereas the others were not. This confirms the result obtained by Mapson (1943) who showed that a glucoreductone solution, after oxidation by iodine and reduction by hydrogen sulphide, behaved as a mixture of reductants, one of which was reduced by hydrogen sulphide more rapidly than the others, and was apparently present to the extent of 35% of the total indophenol reducing titre. The present experiments with *Esch. coli* lead to the same conclusion. Whereas, however,

prolonged treatment with hydrogen sulphide completely restored the whole reducing power of the solution, with *Esch. coli* one only of these reductants was reduced.

A series of experiments was carried out in which each of the constituents of the tap water was added to the glass-distilled water buffer mixture separately. These included calcium and magnesium salts, iron and copper salts, and carbonates and sulphates. None of these had any effect, but when nitrate in a concentration of 0·001 M was added, the resultant effect was similar to that observed with tap water (Fig. 5).

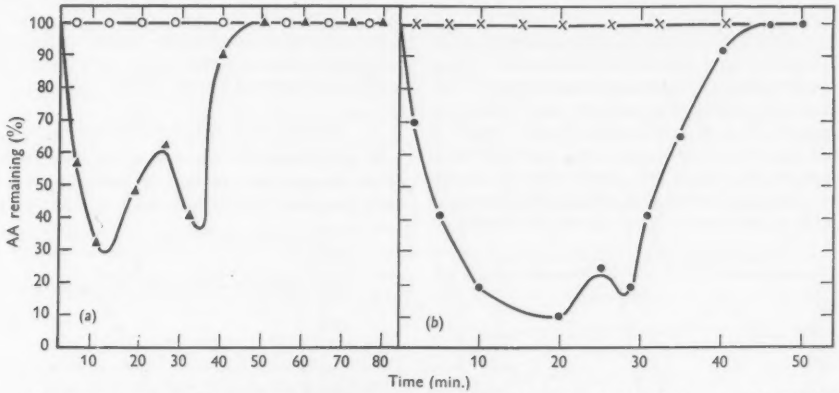

Fig. 4. *a*, the influence of a constituent of tap water on the oxidation of AA by *Esch. coli*. ○—○, 2% HPO_3 buffered to pH 6·2 with NaOH made with glass-distilled water; ▲—▲, 2% HPO_3 buffered to pH 6·2 with NaOH made with tap water. AA estimated by titration in acid solution. *b*, the oxidation of AA in acid solution caused by the action of *Esch. coli* on some constituent of tap water. ×—×, AA estimated by titrations in neutral solutions; ●—●, AA estimated by titrations in acid solution, pH 2·0.

The bacterial reduction method is thus, under these conditions, not as specific for dehydroascorbic acid as was claimed by Stewart & Sharp. It is, however, true that if any of these substances were present in the form of the dehydro derivatives, they would interfere less than in the corresponding chemical method of reduction by hydrogen sulphide.

Interference due to nitrite

When pure ascorbic acid is added to a phosphate buffer solution of pH 6·2, made with glass-distilled water and incubated with or without the bacterial suspension at a temperature of 35° under anaerobic conditions, there is no apparent oxidation of the ascorbic acid when samples are mixed with metaphosphoric acid prior to titration. When the buffer solutions were made with ordinary tap water and the above procedure followed, there was at first an apparent rapid oxidation of ascorbic acid, followed by a rapid regeneration of all of the ascorbic acid, and this despite the fact that dehydroascorbic acid seemed to have been produced in relatively large amounts as indicated by decrease in dye-reducing power (Fig. 4*a*).

This oxidation of ascorbic acid was however only apparent and was caused by the interaction of some constituent in the reaction mixture with ascorbic acid in acid solution, because when the titrations were carried out in neutral solution there was no evidence of any such oxidation (Fig. 4*b*).

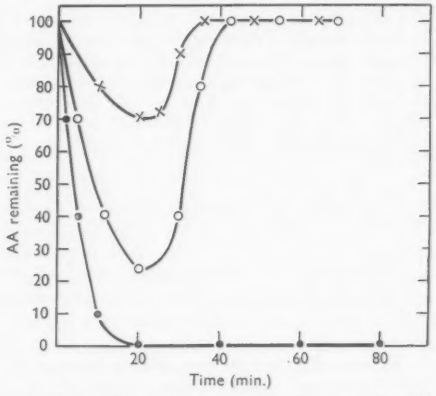

Fig. 5. The oxidation of AA in acid solution produced by a substance formed by the interaction of *Esch. coli* and nitrate. ●—●, 0·01 M-$NaNO_3$; ○—○, 0·001 M-$NaNO_3$; ×—×, 0·0005 M-$NaNO_3$.

That nitrate itself was not the oxidizing agent was shown by the fact that, in acid solution, nitrate does not oxidize ascorbic acid; but the well known fact that *Esch. coli* possesses nitratase (Stickland, 1936),

an enzyme causing the reduction of NO_3^- to NO_2^-, led us to examine whether the production of this substance by *Esch. coli* could be the cause. It can easily be shown that while nitrite will not oxidize ascorbic acid, nitrous acid oxidizes ascorbic acid very rapidly.

We therefore followed the apparent oxidation of ascorbic acid by *Esch. coli* in the presence of added nitrate and examined, in a parallel experiment, the production of nitrite. The results are illustrated graphically in Fig. 6. They show clearly that the apparent loss of ascorbic acid can be attributed quantitatively to the rapid production of nitrite from the nitrate under the anaerobic conditions, and that the loss of ascorbic acid occurs by oxidation

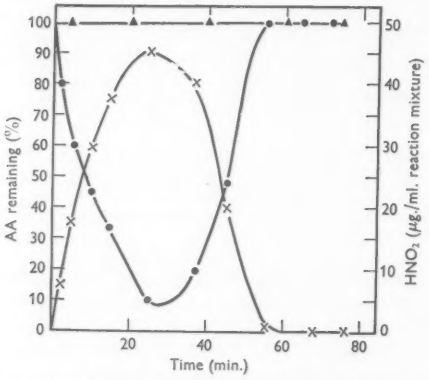

Fig. 6. Correlation between the production of nitrite by *Esch. coli* and the oxidation of ascorbic acid in acid solution. ●—●, ascorbic acid estimated by titration in acid solution, pH 2·0; ▲—▲, ascorbic acid estimated by titration in neutral solution; ×—×, production of nitrite. Reaction mixture contained ascorbic acid 0·080 mg./ml. with 0·001 M-$NaNO_3$ in phosphate buffer pH 6·2.

when the solution containing nitrite is acidified. After the disappearance of the nitrite by the continued activity of the bacteria, there is no further loss of ascorbic acid on acidification of the solution. Moreover, as Fig. 6 shows, there is no oxidation of ascorbic acid if the titrations are carried out in neutral solution.

Similar results to these were obtained with extracts made from fresh potato tissue. Fresh tissue was extracted with 2% metaphosphoric acid in glass-distilled water in a modified Waring blender, a description of which is given by Barker & Mapson (1950). The extract was filtered to remove starch and protein and adjusted to pH 6·2 with sodium hydroxide. When this extract was treated with *Esch. coli* suspension under anaerobic conditions, and samples removed into metaphosphoric acid prior to titration, there was first a rapid fall in the ascorbic acid followed by a regeneration of ascorbic acid within a period of 1 hr. The fall in ascorbic acid was, as in the previous experiments, correlated with the production of nitrite from the nitrate present in the extract, and the rise again in the indophenol titre was due to the disappearance of nitrite. No oxidation of ascorbic acid occurred if the extract was titrated against indophenol dye in neutral solution.

Experiments with dehydroascorbic acid

If similar experiments to those described in the previous section are carried out with dehydroascorbic acid instead of ascorbic acid, the production of

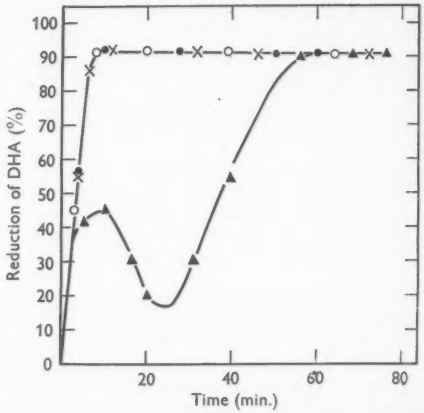

Fig. 7. The reduction of dehydroascorbic acid by *Esch. coli* in the presence and absence of nitrate. ▲—▲, 0·001 M-$NaNO_3$, AA estimated by titration in acid solution pH 2·0; ○—○, 0·001 M-$NaNO_3$, AA estimated by titration in neutral solution; ×—×, no nitrate, AA estimated by titration in acid solution, pH 2·0; ●—●, no nitrate, AA estimated by titration in neutral solution.

nitrite delays the reduction of the dehydroascorbic acid until all the nitrite has disappeared. The results, illustrated in Fig. 7, show that the delay is not real but is an artifact, caused by the oxidation of the ascorbic acid by the nitrous acid formed when the solution is acidified. Only when nitrate is present and when the ascorbic acid formed is estimated by titration in acid solution is there any apparent delay in the reduction of the dehydroascorbic acid. In the absence of nitrate, the same result is obtained whether the titrations are carried out in acid or in neutral solution.

Elimination of interference due to nitrite

(*a*) *Titration in neutral solution.* The simplest way to eliminate interference due to nitrite when estimating ascorbic acid in pure solution is to carry out

the titration in neutral solution. However, when dealing with extracts of biological material, this procedure is not to be recommended for the indophenol method of assay is less specific for ascorbic acid at neutrality and the end point of the titration is much more difficult to determine. Moreover, as pointed out by Stewart & Sharp, if the titration is to be carried out in neutral solution, the reducing action of the bacteria on indophenol dye must be prevented by some means. This can only be done by heat treatment or by the addition of non-acid poisons. Such procedures are more likely to lead to oxidation of ascorbic acid than procedures in which the bacteria are destroyed by the addition of acids such as metaphosphoric which eliminate bacterial activity and at the same time stabilize the ascorbic acid. For these reasons we have investigated other means whereby the interference due to nitrous acid may be removed.

(*b*) *The use of substances reacting with nitrous acid.* Ascorbic acid may be protected from the oxidative effect of nitrous acid by the presence in the solution of substances which react with the latter. The data in Table 1 show the protective effect on ascorbic acid

Table 1. *Protection of ascorbic acid from oxidation by nitrous acid by urea and amidosulphonic acid*

Exp. no.	Solution tested	Ascorbic acid by indophenol titration (mg./ml.)
1	Ascorbic acid in 4% HPO_3	0·063
	Ascorbic acid + 0·001 M-$NaNO_2$ in 4% HPO_3	0
	Ascorbic acid + 0·001 M-$NaNO_2$ + urea 20% (w/v)	0·051
2	Ascorbic acid in 4% HPO_3	0·078
	Ascorbic acid + 0·001 M-$NaNO_2$ in 4% HPO_3	0
	Ascorbic acid + amidosulphonic acid 1% in 4% HPO_3	0·078
	Ascorbic acid + 0·001 M-$NaNO_2$ + amidosulphonic acid 1% in 4% HPO_3	0·078
3	Ascorbic acid in 4% HPO_3	0·087
	Ascorbic acid + 0·001 M-$NaNO_2$ in 4% HPO_3	0·005
	Ascorbic acid + amidosulphonic acid 2% in 4% HPO_3	0·087
	Ascorbic acid + 0·001 M-$NaNO_2$ + amidosulphonic acid 2% in 4% HPO_3	0·087
4	Potato extract in 2% HPO_3	0·0250
	Potato extract + amidosulphonic acid 1% in 2% HPO_3	0·0255
	Potato extract + 0·001 M-$NaNO_2$ + 1% amidosulphonic acid in 2% HPO_3	0·0252

of urea and the more highly reactive amidosulphonic acid, NH_2SO_3H (cf. Brooks & Pace, 1940). Urea in a concentration of 20% (w/v) in metaphosphoric acid only partially protects ascorbic acid from a concentration of nitrous acid of 0·001 M, but amidosulphonic acid in a concentration of 1% (w/v) gives complete protection, and does not itself reduce indophenol dye. Amidosulphonic acid may therefore be added to metaphosphoric acid to eliminate the induced oxidation of ascorbic acid by nitrous acid.

(*c*) *The use of substances inhibiting the nitratase enzyme of* Esch. coli. Confirmation that our interpretation of the observations made in the preceding section of this paper was correct was obtained in the following experiments. Stickland showed that the enzyme, nitratase, is sensitive to cyanide, since the reduction of nitrate to nitrite by *Esch. coli* is suppressed in the presence of 0·001 M-potassium cyanide. In an experiment, the results of which are shown in Fig. 8 in which both nitrate and cyanide were present,

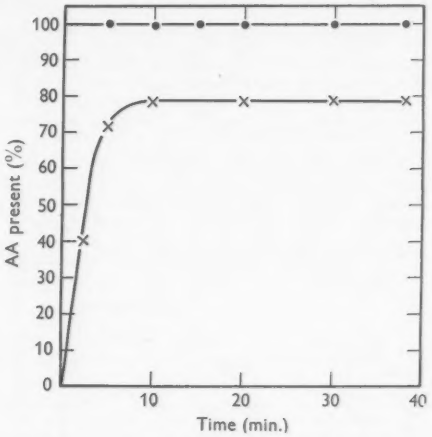

Fig. 8. The oxidation of ascorbic acid and reduction of dehydroascorbic acid by *Esch. coli* in the presence of 0·001 M-$NaNO_3$ and 0·001 M-KCN. ●—●, AA added originally; ×—×, DHA added originally. All estimations carried out in acid solution, pH 2·0.

there was no induced oxidation of ascorbic acid on acidification of the solution and the reduction of dehydroascorbic acid proceeded rapidly. In parallel experiments, in which cyanide and nitrate but no ascorbic acid were added, direct tests showed that the production of nitrite was completely suppressed.

One feature of these experiments is worthy of note; that, despite a rapid reduction of dehydroascorbic acid, the final percentage recovered was lower than in those experiments in which cyanide was absent, Fig. 9. Herein lies the chief objection to the use of cyanide. We have shown by the chemical hydrogen sulphide method that, under the conditions of these experiments (pH 6·2, 35°), the addition of

0·001 M-cyanide, a concentration necessary to inhibit the production of nitrite by the bacteria, accelerates the disappearance of dehydroascorbic acid by converting it into 2:3-diketogulonic acid, and it is presumably to this effect that the lowered percentage recoveries of dehydroascorbic acid are due. Because of this, the use of cyanide is to be avoided.

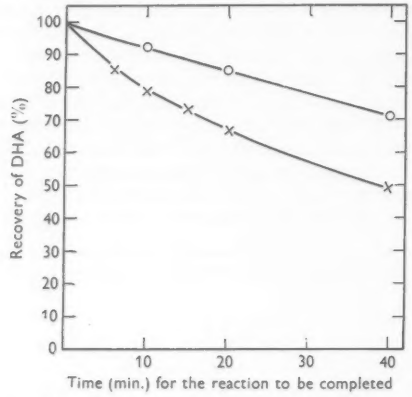

Fig. 9. Percentage recovery of dehydroascorbic acid as ascorbic acid by reduction by *Esch. coli* in presence and absence of KCN (0·001 M) with glucose 1% (w/v) as substrate. ○—○, no KCN; ×—×, 0·001 M-KCN.

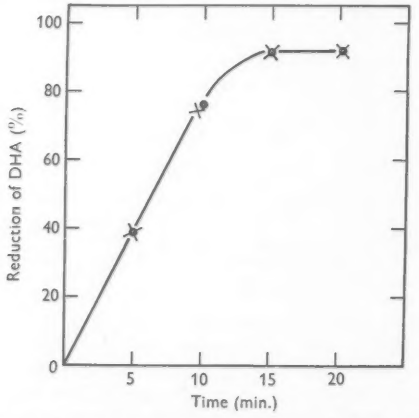

Fig. 10. Reduction of dehydroascorbic acid by *Esch. coli* in the presence of 0·001 M-$NaNO_3$ and 0·001 M-NaN_3 compared with the reduction in absence of nitrate or azide. ×—×, nitrate + azide; ●—●, control. All estimations of AA in acid solution, pH 2·0.

In our search for an alternative inhibitor, we found that azide, in a concentration of 0·001 M, will, like cyanide, effectively inhibit the conversion of nitrate to nitrite by *Esch. coli*, without affecting the enzymic systems associated with the reduction of dehydroascorbic acid. Moreover, it possesses the advantage over cyanide that it does not affect the rate of conversion of dehydroascorbic acid to 2:3-diketogulonic acid. The result of an experiment illustrated in Fig. 10 shows the same rapid reduction of dehydroascorbic acid and the same percentage recovery of dehydroascorbic acid, as ascorbic acid, with azide in the presence of added nitrate, as in the control solution in which neither azide nor nitrate were present. Further, in other experiments in which ascorbic acid was added with azide and nitrate, there was no oxidation of the ascorbic acid on acidification of the solutions, showing that no nitrite had been produced.

The use of the method with plant extracts

Our experience of the method of estimating dehydroascorbic acid in plant tissues has so far been confined to potato. Good recoveries of dehydroascorbic acid added in known amounts to extracts of this tissue have been obtained. These results will be reported in a forthcoming paper. One possible source of loss of dehydroascorbic acid has, however, been noted. After extraction of the tissue with metaphosphoric acid, dehydroascorbic acid may be lost during the neutralization of the extract to pH 6·2 preparatory to adding the bacterial suspension. Such a loss invariably occurs when the extract is neutralized with sodium hydroxide. This is due to the practical difficulty of preventing parts of the solution from becoming temporarily alkaline, with consequent rapid destruction of dehydroascorbic acid. Losses as high as 40% have been observed from this cause. Such losses may, however, be prevented if the metaphosphoric acid extract is neutralized with a solution of potassium hydrogen phosphate (40%, w/v) added slowly, with constant agitation.

SUMMARY

1. The reduction of dehydroascorbic acid by *Escherichia coli* has been reinvestigated. Complete recovery of dehydroascorbic acid as ascorbic acid was not obtained owing to small concurrent loss of dehydroascorbic acid by conversion to diketogulonic acid. Loss due to this cause can, however, be calculated and allowed for and dehydroascorbic acid may accordingly be estimated quantitatively by this method.

2. The difficulty associated with this method of estimating dehydroascorbic acid in vegetable extracts, reported by earlier workers, has been shown to be due to the reduction of nitrate to nitrite by *Escherichia coli* under anaerobic conditions. On acidification, the nitrous acid formed rapidly oxidizes ascorbic acid.

3. Interference in the estimation of ascorbic acid by nitrous acid may be overcome by the use of amidosulphonic acid, which reacts rapidly with nitrous acid and so protects ascorbic acid from oxidation.

4. The nitratase enzyme of *Escherichia coli* may be completely inhibited by potassium cyanide or sodium azide, without affecting the enzyme systems associated with the reduction of dehydroascorbic acid.

5. The presence of potassium cyanide in a concentration of 0·001 M accelerates the conversion of dehydroascorbic acid to diketogulonic acid, but sodium azide in a similar concentration has no effect. Sodium azide may, therefore, be used to prevent the formation of nitrite by *Escherichia coli* without any disadvantageous effect on the reduction of dehydroascorbic acid to ascorbic acid.

6. The rate of reduction of the oxidized forms of other enediols has been studied. These include D-*iso*ascorbic acid, hydroxytetronic acid, reductic acid, reductone (enol of tartronaldehyde) and the reductants present in a glucoreductone solution. All these substances with the exception of reductone were reduced, but at a slower rate than dehydroascorbic acid.

We wish to thank Dr R. H. M. Robinson for his help in preparing the bacterial suspensions, Dr J. Barker for his interest and advice, and Mr D. Wardale for his technical assistance in carrying out the work.

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The Stimulant Involved in the Germination of *Orobanche minor* Sm.

1. ASSAY TECHNIQUE AND BULK PREPARATION OF THE STIMULANT

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Among the parasitic plants, the subfamily Rhinanthoideae and the closely related Orobanchaceae are unusual in that all the species of these groups are root parasites. Those of the former group have green leaves and are only partially dependent upon other plants, but the Orobanchaceae are complete parasites which lack chlorophyll and have a much reduced and modified structure. They consist of little more than an erect flowering shoot above the ground and they produce many thousands of minute seeds, each no more than about 0·3 × 0·2 mm. in external dimensions. The base of the shoot is attached below ground to the root system of the host plant.

A number of species of *Orobanche* have considerable economic importance in that they damage a variety of crops, particularly in Mediterranean climates. Even in this country, *O. minor*, the lesser broomrape, may cause partial or complete failure of clover. The seeds of many species of these parasites normally germinate only when they are in the immediate vicinity of a host root. According to Lindley (1853) this condition was first demonstrated by Vaucher with *Orobanche*. The phenomenon was subsequently investigated by Koch (1887) again with *Orobanche*, by Heinricher (1898) with *Lathraea* and by Pearson (1912) with *Striga*.

It has been shown that the influence of the growing root is due to a chemical stimulant which is released from it and which is required by the seed in germination. Saunders (1933) induced germination in *Striga lutea* seeds by irrigating them with water that had percolated through sand containing growing maize roots, and Barcinsky (1934) and Chabrolin (1934) reported similar results with *Orobanche cumana* and *O. speciosa* respectively. It was further shown by these two workers that a dry residue could be obtained from aqueous extracts of roots and other parts of host plants which, when redissolved in water, promoted the germination of the parasite seed.

The chemical nature of the stimulant for *Striga* is being investigated in these laboratories and Brown, Johnson, Robinson & Todd (1949) have reported that a crude concentrate of the natural stimulant contained sugars and that certain pure sugars promoted the germination of the seed. The present paper is the first of a series in which the results of a parallel investigation on the stimulant for *Orobanche* will be reported.

In the first place, the range of stimulating organisms was investigated in order to decide the most suitable source of the germination factor. Not all of the species of *Orobanche* are narrowly host specific; thus whereas *O. hederae* is normally restricted to ivy (it has been reported on a few other members of the Araliaceae), other species such as *O. speciosa*, *O. cumana* and *O. minor* parasitize a wide range of hosts. *O. minor* Sm. with which this investigation is concerned has a particularly wide host range, and has been reported as occurring on more than sixty different hosts.

In the total host-parasite relationship two phases must be recognized. In the first the host root stimulates the seed to germinate and induces it to produce an independent seedling; in the second the seedling becomes attached to the root of the host and thereafter exists parasitically upon it. The restriction of host species (at least with *O. minor* and *O. speciosa*) refers in many instances to the second phase; during the first, stimulation can probably be given by the roots of a much wider range of species, and it can be given by roots which are never parasitized. Chabrolin (1934, 1935) showed that *O. speciosa*, for which a stimulant is produced by *Vicia faba* and other plants which are parasitized, may also be germinated by the roots of *Cicer arietinum*, *Linum usitatissimum* and a number of other species which are not. The significance of this phenomenon in relation to angiospermous parasites as a whole has been discussed by Brown (1945).

A comparison of clover, maize, sorghum and linseed has led us to adopt linseed (var. Blue-flowering Plate) as the stimulant source in this investigation.

The bulk preparation of the stimulant has been carried out by an extension of the method used by Brown *et al.* (1949) for the preparation of the *Striga* factor. The roots of intact linseed seedlings were grown in water and the active material adsorbed from the aqueous solution by purified activated charcoal. The subsequent elution and concentration of the factor is described in the succeeding papers and most of the present communication is devoted to a description of the assay technique employed. This technique is also based on that developed by Brown *et al.* (1949) for use with *Striga*. The procedure, however, has been further elaborated and certain modifications have been introduced that are required by the peculiarities of the germination process in *Orobanche*.

The assay depends on the condition that percentage germination decreases with increasing dilution of a stimulating solution. Different samples of seed or the same sample at different times, however, vary in the magnitude of their response to the stimulant and the results obtained with any given experimental preparation must therefore be correlated with the results obtained with a standard solution. These variations in seed behaviour, which involve both the maximum percentage germination which may be attained and the concentration of stimulant required to produce a particular percentage germination, are profoundly affected by certain treatments applied before the seeds are exposed to stimulant solutions. The assay technique involves, therefore, preparation or pretreatment of the seed, the actual germination procedure, and the preparation of the standard fluids. Each of these aspects is described separately in the experimental section. The treatment of the assay technique is completed by descriptions of the general design of a single assay and by a consideration of the errors of the method.

EXPERIMENTAL

Assessment of various stimulating organisms

Conditioned seeds were sprinkled over a sheet of moist filter paper laid against a vertical sheet of glass resting in a shallow trough of water. Evaporation from the filter paper was limited by securing a second sheet of glass at a distance of 3 mm. from the first and separated from it at the vertical edges with a wax seal. Seedlings with their roots between the two sheets of glass were placed along the upper edges of the two sheets. The roots grew downwards over the surface of the filter paper carrying the *Orobanche* seeds. The effects of the roots of clover, maize, sorghum and linseed were investigated. It was found that the most vigorous germination was given by linseed and the least by clover. Maize and sorghum gave effects intermediate between those of the other two. It may be noted that seedlings of maize, sorghum and linseed which are not normally parasitized gave a stronger stimulating effect than those of clover which is a normal host.

The effect of linseed was striking and well defined. Whereas with the other species germination was restricted to a zone a few centimetres wide along the course of the stimulating root, with linseed germination after 7 days was observed over the whole surface of the filter paper which covered an area 8×10 cm. With other techniques the stimulating effect of roots of pelargonium, ivy, wheat, barley, oats, potato and willow was investigated. Stimulation was observed with all these, but in no case was it as great as with linseed.

Bulk preparation of the stimulant

Seeds (150–200) of linseed were germinated on pieces of muslin each laid on a sheet of filter paper moistened with a few drops of a dilute calcium hypochlorite solution in shallow dishes kept in the dark in a constant temperature room at 25°. The seeds germinated in the dishes and on the

3rd day each net carrying growing seedlings was stretched over a frame of glass tubing and supported on the surface of 1–1·5 l. of distilled water in an aluminium bowl. One hundred bowls were set up each week, and the cultures were continued at 25° in the dark for a further 5 days, at the end of which time the undiluted fluid in the bowls promoted the full germination capacity of the parasite seeds.

The solution was treated with charcoal (4·25 g./l.) which was allowed to settle, filtered off and formed into a dry cake by removal of water in a press. After the charcoal treatment the filtrate was biologically inactive and was discarded. The bulk preparation of the stimulant was carried out at Leeds, and the active material sent to Cambridge, after adsorption on to the charcoal.

Considerable difficulty has been experienced in obtaining suitable grades of charcoal for this work and even with the grade finally selected (Sutcliffe, Speakman and Co., Genster charcoal) preliminary purification was necessary. The charcoal (500 g.) was washed successively with water, methanol, ether, ethyl acetate, acetone and finally water (500 ml. of each) and then separated and dried as far as possible by suction in air when it still contained adsorbed water (65–70 g.). After removal of the solvent from the combined extracts from the charcoal, the water-soluble fraction (357 mg.) of the residue (853 mg.) contained phosphate and sulphate ions but no chloride and was strongly acidic. It showed inhibitory effects on the growth of germinating *Orobanche* seedlings. The inhibition was particularly marked on the epidermis of the root and gave an appearance similar to that described by Burström (1949) for the inhibiting effect of di-*n*-amylacetic acid on the growth of wheat roots.

ASSAY TECHNIQUE

Pretreatment of the seed

Chabrolin (1938) reported that with *Orobanche* more vigorous germination is obtained when the seeds are stored moist before the stimulant is applied to them. Brown & Edwards (1944) found with *Striga* that with a standard solution percentage germination increased as the period of incubation at 25° under moist conditions was prolonged over a period of 14 days. A similar condition has been observed by us with *Orobanche*. The change in germination capacity with time of pretreatment is shown by the data of Fig. 1 which were obtained by placing seeds on a moist sintered-glass disk in a Petri dish, incubating them at 25°, removing samples at intervals and treating them with a standard solution. It is evident that under the conditions of the experiment germination increases over the first 14 days and unlike the position with *Striga* thereafter remains more or less constant. Fig. 1 shows only results obtained up to the 32nd day. We have, however, examined a culture over a period of 350 days, and noted only a slight decrease in germination capacity from the 14th day to the end of the experimental period. The germination capacity of other cultures has, however, often decreased more rapidly and the origin of this variability is being investigated.

The change in germination capacity over the first 14 days of pretreatment is clearly an important condition for the assay and in the normal routine, pretreatment dishes were set up at intervals and seeds were taken from them only after they had been incubated for at least 21 days.

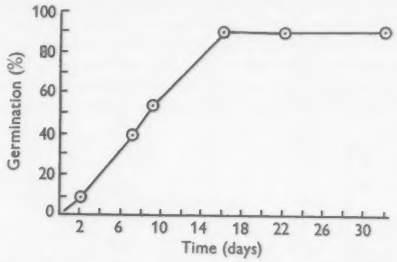

Fig. 1. Effect on percentage germination of incubating seed in moist condition at 25° before application of a standard stimulant solution. Time along abscissa refers to period of incubation.

All the seed used in this investigation belonged to the species *O. minor* Sm., most of which was obtained from a badly infested clover field near Canterbury in August 1948. We are deeply indebted to the staff at the Royal Botanic Garden, Kew, for help in the identification of the species.

Germination test

Percentage germination was determined in hanging drop cultures. This technique is that originally adopted by Brown & Edwards (1944) with *Striga*, since it is the most convenient method for incubating and observing seeds as small as those of *Striga* and *Orobanche*. But whereas Brown & Edwards used single drop cultures, we have used a multiple drop technique by which 25 drops, which represented five replicates for each of five solutions, were incubated within a single flat chamber, which was formed from two glass plates each about 8 cm. square separated by a square frame of glass rod about 3 mm. thick and closed along the edges with a mixture of equal parts of vaseline and embedding wax. The 25 equally spaced drops hung from the lower surface of the upper sheet of glass.

The cultures were set up in the following manner. A glass plate was placed over a sheet of paper of the same size divided into 25 equal squares and the exposed surface of the plate was rubbed with a cotton wool pad charged with liquid paraffin. Five drops (each 0·01 ml.) of an experimental solution were then delivered each above the centre of one of the squares on the paper in a horizontal row, the other four rows being treated similarly each with a different solution. Enough seed for a complete experiment was transferred from the pretreatment dish to a separate vessel, and from this to the drops by means of a platinum spiral which formed a short cylinder into which the seeds were pressed. The spiral

carrying the seeds was brought lightly into contact with each drop and delivered into it 25–50 seeds, approximately the same number of seeds being placed in each drop. The platinum spirals were carefully flamed before they were used for each set of transfers. When the 25 drops had been charged with seeds, the plate on which they rested was covered by a lid formed from a second sheet of glass to which a rod frame had been attached with the wax mixture. The sandwich-like unit thus formed was made into a closed chamber by a second wax seal established between the plate carrying the drops and the glass rod frame. The culture chamber was finally inverted with a swift circular motion of the hand bringing the drops to the lower surface of the upper sheet of glass.

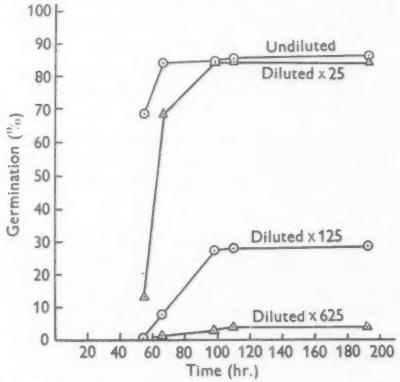

Fig. 2. Change in percentage germination with time after application of an undiluted standard solution, and of solutions diluted × 25, × 125 and × 625.

This multiple drop technique is more convenient than the single drop method since it is considerably more rapid. The trace of paraffin on the surface from which the drops hang prevents them from running together and water does not distil from the drops on the lower plate when the cultures are placed on a metal shelf in the incubator.

After incubation for 4 days at 26° the cultures were examined microscopically, and the total number and the number of seeds that had germinated in each drop were counted and the percentage germination calculated from the totals in each category in the five replicate drops for each treatment.

It has been found that 26° is the optimum temperature for germination and that 4 days are usually required for maximum germination to be attained at this temperature. The change in percentage germination after the 2nd day is shown by the data of Fig. 2. It is evident that in this series of observations the maximum was reached after about 96 hr. Certain cases have been observed in which there was a slight increase after 96 hr., but seed showing this characteristic was not used in the assay.

Preparation of the standard

In this connexion a standard similar to that used in the *Striga* investigation has been adopted. It was prepared by growing 20 seedlings of linseed on a muslin float on the surface of distilled water (50 ml.) in a stoppered jar incubated for 5 days at 26°. In certain circumstances the stimulant is highly unstable and fresh preparations were therefore made at frequent intervals.

It is extremely difficult to secure the same growth in twenty seedlings on different occasions and we have reason to believe that our standard varies slightly. Recently, in order to secure greater uniformity the standard has been prepared from the pooled fluids from four jars cultured simultaneously and further work on this aspect is proceeding.

General design of an assay

The assay involves the determination of germination capacity with a series of dilutions of a standard, with a series of dilutions of an experimental fluid, and with water, the experimental fluid normally being one in which the solute concentration is known. A series with water alone is normally included in the assay, not as with *Striga* in order to assess the percentage of independent germination, but as a check on the independence of the drops in the multiple hanging-drop chamber. If the drops creep laterally, then those with stimulant in them should affect those without it. With *Orobanche*, unlike the position with *Striga*, independent germination is rare.

The results of a typical assay are shown in Fig. 3 and Table 1.

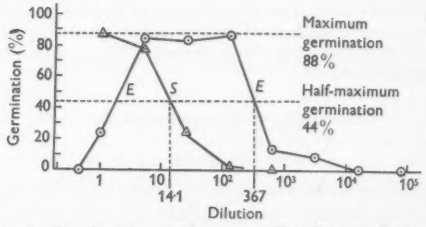

Fig. 3. Results of a single assay. *E* indicates change in percentage germination with dilution of an experimental solution, the R.A. of which is being determined. Figures along abscissa indicate dilution of an original solution. Solution *E* at 1 has a concentration of 1 mg./1 ml. *S* indicates change in percentage germination with dilution of a standard fluid.

Quantitative scales. Two quantities, relative activity (R.A.) and percentage recovery, have been used in this investigation. Relative activity is a measure of the germination-promoting capacity of any preparation, and is defined as the highest dilution of a solution having a concentration 1 mg./ml. which gives half the percentage germination promoted by the undiluted standard. Frequently, the initial concentration of the experimental solution is not 1 mg./ml. and in such cases an appropriate proportionate adjustment is applied to the 'half-value'. The term 'highest dilution' is used, but, as with the case shown in Fig. 3, the undiluted fluid may give negligible germination, and this may first increase and then decrease as the dilution is continued. The results of the assays are plotted and the

Table 1. *Seed counts involved in the assay illustrated by Fig.* 3

Fluid tested	Dilution	Seed counts in each of the five drops No. germinated/Total no. of seeds					Totals	Germination (%)
Water	—	0/25	0/32	0/35	0/29	0/38	0/159	0
Water	—	0/40	0/36	0/27	0/37	0/34	0/174	0
Standard fluid	1*	30/31	27/34	21/27	30/32	31/34	139/158	88·0
	5	21/24	22/25	28/39	32/38	28/43	131/169	77·5
	25	11/21	7/33	6/26	6/25	4/35	34/140	24·3
	125	2/18	0/25	0/36	1/30	0/26	3/135	2·2
	625	0	0	0	0	0	0	0
Extract no. 026 A. at a concentration of 1 mg./ml.	1/2·7†	0	0	0	0	0	0	0
	1*	6/28	17/33	13/35	1/29	0/29	37/154	24·0
	5	34/36	27/31	38/41	28/37	32/42	159/187	85·0
	25	26/30	21/27	17/21	16/18	27/32	107/128	83·6
	125	27/30	27/31	30/38	41/46	41/47	166/192	86·5
	625	13/28	3/38	14/53	3/38	12/35	45/192	23·4
	3,125	3/16	0/20	1/22	1/21	4/26	9/105	8·6
	15,625	0	0	0	0	0	0	0
	78,125	0	0	0	0	0	0	0
	390,625	0	0	0	0	0	0	0

* Undiluted. † Dilution 1/2·7≡concentration of 2·7 mg./ml.

Maximum germination in undiluted fluid, 88 %.

The half-values (the dilution promoting 44 % germination) determined by interpolation arithmetically or graphically: standard fluid, 14·1; extract no. 026 A, 367.

Factor for seed sensitivity = 50/14·1.

'Relative activity' of extract no. 026 A = 367 × 50/14·1 = 1300.

R.A. values found by interpolation. For the data of Fig. 3 the value is 367. This, however, is a crude figure to which a further correction must be applied to correct for the variability of the seed.

Ideally, the assays should be made with a strain of known genetical constitution, and with seed in a constant metabolic state. In this instance, however, these requirements cannot be fulfilled since the seed can only be obtained in sufficient quantity from a wild population and only at one period of the year. During storage, after-ripening changes no doubt occur and further, the conditions during pretreatment have not been completely standardized. As a result of these several uncontrolled variables, the reaction of the seed used in the assay is not constant, so that the same percentage germination with different samples of seed may be given with different concentrations of the stimulant.

The correction is based on the results given by a series of dilutions of the standard fluid, and is again based on the 'half-value'. With the seed of Fig. 3 the half-value is given with a dilution of 14·1, but usually it is given at a dilution of 50, and this value is taken as an arbitrary standard from which all the corrections are calculated. Clearly seed giving a half-value with the standard of 14·1 is

likely to give a lower R.A. figure than one giving 50. Thus the correction involves a proportionality factor, in this instance of 50/14·1. The crude R.A. figure is multiplied by this factor to give the corrected R.A., which for the data of Fig. 3 is 1300.

It is probable that the errors involved in the R.A. determination are large. When the same sample of seed has been used in duplicate determinations, the error does not exceed 100 %. It may, however, be as great as 300% when the results involve several assays prepared on different occasions with different batches of seed, such as the data involved in following the amounts of stimulant recovered at various stages in a series of operations designed to concentrate and to purify the natural stimulant. Although the percentage errors are large it may be emphasized that the R.A. scale ranges from 1 to about 10,000,000, and the range is sufficiently large to enable large differences in activity to be evaluated.

The percentage recovery provides a figure for the proportion of the natural stimulant that has been retained in any purification procedure. Thus if a solution (total volume V ml.) with corrected half-value X yields purified material (W mg.) with R.A. Y then the percentage recovery is 100 WY/VX. The errors in the estimation of percentage recovery are likely to be larger than those of the R.A. determination since they involve the errors of at least two independent estimations. Occasionally, the percentage recovery exceeds 100 and these excessively large values must be attributed either to the removal of inhibitory material or to the errors of estimation.

SUMMARY

1. *Orobanche minor* is an angiospermous parasite, and the stimulant that the seed requires for germination may come from a variety of species. Linseed which is being used as the source of the germination factor in this investigation is not normally parasitized by *Orobanche minor*.

2. The bulk preparation of the stimulant is described. The method involves growing the roots of seedlings in water, from which the stimulant is recovered by adsorption on to charcoal.

3. The techniques that are being used for the determination of the 'relative activity' of various preparations, and for the calculation of percentage recovery of the stimulant during purification are described.

It is a pleasure to record our thanks to Prof. A. R. Todd, F.R.S., for his continued interest and advice throughout this work. We are grateful to the Agricultural Research Council for providing financial support for the development of this investigation at Leeds and our thanks are also due to Glaxo Laboratories Ltd. for a grant to one of us (A. G. L.).

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The Stimulant Involved in the Germination of *Orobanche minor* Sm.

2. CHROMATOGRAPHIC PURIFICATION OF CRUDE CONCENTRATES

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(*Received* 9 *September* 1950)

The procedure adopted for the adsorption of the chemical factor responsible for the germination of *Orobanche minor* from aqueous solutions by means of active charcoal has been described in the previous paper (Brown, Greenwood, Johnson & Long, 1951), where the technique adopted for the biological assay was also described. Solutions of the stimulant were unstable under certain conditions, as evidenced by loss of biological activity, so that it was apparent that the design of any purification procedure would depend on the nature and extent of this decomposition. The stability of solutions of the stimulant under various conditions of temperature and pH have therefore been examined. The experiments have been carried out with the standard assay fluid (Brown *et al.* 1951) which, when withdrawn from the

culture jars, is at approximately pH 5. Samples of this solution stored at 4° without adjustment of pH have been found to retain their stimulating capacity with very little or no loss over a period of 4 months. On the other hand, at pH 9 there was a rapid decrease in the stimulating capacity. The rate of inactivation was greater at higher temperatures at all pH levels, and especially so under alkaline conditions (Table 1). It was also observed that on heating alkaline solutions of the stimulant there was an appreciable decrease in the pH (Table 2), but no such change was obtained in solutions with pH < 5. These experiments indicated that the solutions of the factor were most stable between pH 3 and 4, and that alkaline treatment and excessive heating would have to be avoided throughout the isolation.

Several solvents have been examined for the elution of the stimulant from the charcoal, and the results of a series of comparative experiments are shown in Table 3, from which, on the basis of the 'Relative activity' (R.A.) (Brown *et al.* 1951) and the percentage recovery figures, it will be apparent that elution with chloroform-methanol is the preferred method. This has been used in most of the subsequent work. The inactivation of the stimulant by prolonged heat treatment has already been described, and it is therefore essential that the removal of solvent from solutions should be carried out at as low a temperature as possible. Preliminary freeze-drying of the charcoal before extraction with solvents seems, however, to offer no advantage.

The material as eluted from the charcoal was undoubtedly very crude, and a variety of techniques has been investigated for the purification of concentrates prior to chemical degradative studies. The most promising results have come from separations based on partition chromatography, described in the present paper, and those based on extraction methods which will be discussed in a later paper.

Examination of the eluates from the charcoal by paper-strip chromatography (Consden, Gordon & Martin, 1944), using *n*-butanol-acetic acid-water (Partridge, 1948) as the solvent system, has shown that the greatest part of the total activity is to be found in the fractions which travel very close to the solvent front and which are quite distinct from the fractions containing carbohydrates. This was established by drying the completed chromatogram and then cutting it into horizontal strips of convenient dimensions. Each of these was extracted with water and the individual extracts were then tested for biological activity. The results of typical experiments are shown in Figs. 1 and 2, the former being the chromatogram of an acetone eluate and the latter that of a chloroform-methanol eluate. The 'tailing' effect of the biologically active material in Fig. 1, which is not so apparent in Fig. 2, is probably caused by the larger amount (24 mg.) used in the former chromatogram as compared with that (5 mg.) used in the latter.

The zones near the solvent front containing the active fraction appeared as weakly absorbing bands when the chromatograms were used as filters between an ultraviolet lamp and photographic paper (Markham & Smith, 1949), and it was also noted that these zones reduced Tollens reagent when this was sprayed on to the paper.

Brown, Johnson, Robinson & Todd (1949) have shown that certain sugars induce germination in *Striga*, and that sugars are present in the concentrates of the germination factor prepared from the *Sorghum* host. *Orobanche* also responds to *Sorghum* preparations, although less vigorously than to those from linseed cultures, and an examination was therefore made of the nature of the sugar content of the acetone eluate from the linseed preparations. The presence of reducing sugar in the crude concentrate was indicated by a Molisch test and by the ready reduction of Fehling solution. A reducing sugar determination (Somogyi, 1945) gave a value corresponding to 5–10 % (calculated as glucose) of the crude extracts. The individual sugars were separated by means of paper chromatography, seven compounds being detected, of which two were identified as glucose and arabinose and a third possibly as 2-ketogluconic acid. The implications of the presence of 2-ketogluconic acid will be discussed in a later paper.

The chromatograms were also examined for components other than carbohydrates. Amino-acids were absent (ninhydrin reaction), but two phosphate-containing fractions (R_F 0·13 and 0·29) were detected by spraying with a reducing molybdate solution under special conditions (Hanes & Isherwood, 1949). No reactions on the paper were observed with ferric chloride or diazotized sulphanilic acid.

Simple chromatography on neutral alumina proved to be of little value for the purification of larger quantities, as the stimulant was too strongly adsorbed and could not be eluted. However, in view of the separation of active material from carbohydrates achieved by paper-strip chromatography, larger scale chromatography on columns of cellulose has been used with a degree of success. Nearly all of the active material was contained in the fractions which moved very close to the solvent front, and the product obtained from these fractions after removal of the solvent gave R.A. values of $1–2 \times 10^5$. There was also little loss of total activity. Data for typical chromatograms are shown in Tables 4 and 5.

In the course of this work, a wide variety of synthetic compounds has been tested for activity towards *Orobanche* seeds. None has so far been found to be active in the concentration range $1/10^3$

to $1/10^6$, and in this respect the situation with *Orobanche* differs from that observed with *Striga* (Brown *et al.* 1949) where certain sugars were found to be active.

The following compounds have been examined: laevulinic acid, β-ketoadipic acid, catechol, *o*-benzoquinone, cinnamic acid, heteroauxin, sucrose, D-fructose, D-glucose, D-xyloketose, D-glucosone, D-glucuronic acid, D-2-ketogluconic acid and Ca salt, D-5-ketogluconic acid and Ca salt, methylglyoxal, DL-malic acid, fumaric acid, succinic acid, citric acid, pyruvic acid, adenylic acid, L-cysteine, glutathione, *p*-aminobenzoic acid, vitamin B_1, nicotinic acid, calcium pantothenate, β-alanine, vitamin C, coumarin, anhydrotetronic acid, aconic acid, 3-hydroxycoumarin, 3-acetonylcoumarin, homoparaconic acid, patulin, α-methyltetronic acid, 7-hydroxy-4-methylcoumarin, α:β-penteno-δ-lactone, α:β-angelicalactone, α:β-hepteno-γ-lactone, γ-(spirocyclohexyl)-α:β-buteno-γ-lactone, α-(4-hydroxy-3-methoxybenzylidene)-γ-methyl-β:γ-buteno-γ-lactone (Marrian, Russell & Todd, 1949).

EXPERIMENTAL

Stability of the stimulant. The standard fluid (10 ml.; Brown *et al.* 1951) was placed in each of six Pyrex tubes and adjusted electrometrically by the addition of either dil. NaOH or HCl to a range of pH values (0·96–8·8). The tubes were heated at 100° in a water bath and the stimulating capacities of the samples were measured at intervals (Table 1). Observations were also made on the change in acidity with time in the several series, and these results are shown in Table 2.

Table 1. *Rate of inactivation of standard fluid at different* pH *levels at approximately* 100°

(Data represent 'half-values', see Brown *et al.* 1951.)

Time (hr.)	Initial pH						
	0·96	3·62	4·37	6·18	6·85	8·02	8·83
0	50	50	50	50	50	50	50
2	<2	30	17	5	1	0	0
5	0	22	14	2	0	—	0
20	—	9	6	—	0	0	0
50	—	2	1	1	—	—	—
80	—	1	1	1	—	—	—
100	—	1	0	0	—	—	—

Table 2. *Change in acidity during heating of standard fluid at* 100° *with different initial* pH *levels*

Time (hr.)	pH values						
0	0·96	3·62	4·77	6·18	6·85	8·02	8·83
2	0·96	3·64	4·92	5·92	6·72	6·85	7·05
100	—	3·59	4·68	4·98	6·00	6·20	6·85

Elution of the stimulant from charcoal. The results of a variety of elutions with different solvents are shown in Table 3. Methanol was added to a number of the water-

Table 3. *Formation and properties of crude eluates from charcoals given with various solvents*

	Solvent	Weight of charcoal (g.)	Weight of eluate (mg.)	Appearance	Weight of water-soluble fraction of eluate (mg.)	Relative activity	Recovery (%)
(*a*)	Acetone	5	4·9	Yellowish glass	4·9	100	1
(*b*)	(i) Ether (50 ml.) and methanol (20 ml.) (ii) Ether (50 ml.) and methanol (6 ml.) (iii) Ether (3 × 50 ml.)	27	18·8	Nearly colourless glass	15·9	31·6	1·9
(*c*)	Ether (cold) (3 × 50 ml.)	35	31·9	Nearly colourless glass	14·3	1,500	14·3
(*d*)	Ether (hot) 8 hr. constant extraction	35	42·3	Nearly colourless glass	24·3	300	5
(*e*)	(i) Chloroform (50 ml.) and methanol (20 ml.) (ii) Chloroform (50 ml.) and methanol (6 ml.) (iii) Chloroform (3 × 50 ml.)	27	21·2	Nearly colourless glass	13·1	18,000	90
(*f*)	Pyridine (5 × 30 ml.)	15	67·3	Dark-brown oil	67·3	1,770	46
(*g*)	(i) Ethyl acetate (30 ml.) and methanol (10 ml.) (ii) Ethyl acetate (30 ml.) and methanol (3 ml.) (iii) Ethyl acetate (3 × 30 ml.)	15	9·9	Nearly colourless glass	5·6	18,000	70
(*h*)	(i) *n*-Butanol (30 ml.) and methanol (10 ml.) (ii) *n*-Butanol (30 ml.) and methanol (3 ml.) (iii) *n*-Butanol (3 × 30 ml.)	29	12·2	Nearly colourless glass	9·9	4,800	22
(*j*)	Acetone (50 ml.) and ether (20 ml.) (× 5)	29	14·7	Nearly colourless glass	5·2	14,400	64

immiscible solvents in order that the efficiency of the extraction of the wet charcoal should be increased. The extracts were clarified by filtration through Supercel (Johns-Manville Co. Ltd.) and for the biological assay the crude concentrates were extracted with water (5 ml.).

Distribution of the stimulant on paper-strip chromatograms

The acetone eluate (333 mg.) was extracted with dry methanol, the insoluble inorganic salts separated and the solvent removed from the filtrate. The residue (122 mg.) was dissolved in water (2 ml.), and a portion of this solution (0·4 ml. containing 24·4 mg. concentrate) applied to a Whatman no. 1 filter-paper strip (49 × 17 cm.) and developed during 24 hr. with the *n*-butanol-acetic acid-water system (Partridge, 1948), after which the solvent front was 38 cm. in advance of the starting line.

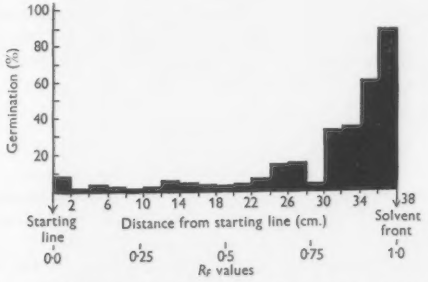

Fig. 1. Histogram showing distribution of stimulant on a paper-strip chromatogram of an acetone eluate after development with *n*-butanol-acetic acid-water solvent mixture.

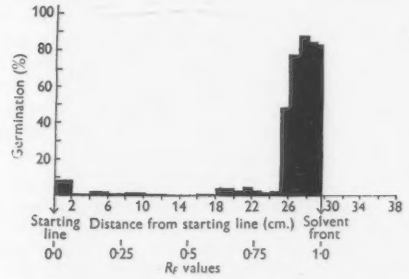

Fig. 2. Histogram showing distribution of stimulant on a paper strip chromatogram of a $CHCl_3$-methanol eluate after development with *n*-butanol-acetic acid-water solvent mixture.

A similar experiment was conducted with a portion (1 ml. containing 5 mg. of concentrate) of an aqueous extract of the material obtained from a $CHCl_3$-methanol eluate. After development of the chromatogram as in the previous experiment, the solvent front was 29·5 cm. in advance of the starting line. The papers were then dried *in vacuo* at room temperature. In an initial experiment the distribution of the stimulant on the paper was explored by allowing seeds of *Orobanche minor* to germinate in contact with portions of the actual paper, but it was later found that more vigorous germination was obtained in hanging drop cultures with fluids prepared by extracting portions of the chromatogram with water. This method has the additional advantage that the technique of the test is essentially the same as that used in the assay of stimulant preparations. The chromatograms were therefore divided into a number of strips parallel to the solvent front, each strip was eluted with water for several hours and the percentage germination in each of the fluids so obtained was found from the totals in five replicate drops of each fluid mounted in the usual culture chambers and incubated at 26° for 4 days. In another experiment the procedure of preparing the chromatogram was followed, but without using any stimulant material. The paper was cut up and extracted in the usual way, but no germination was recorded from any part of the paper.

In the experiment represented diagrammatically in Fig. 1, the chromatogram from the starting line to the solvent front was cut into 19 strips each 2 cm. wide. Each strip was soaked overnight in distilled water (2 ml.) at approximately 4° and the fluids tested in the manner described above. In the case of the chromatogram of the $CHCl_3$-methanol eluate, narrower strips were taken in the biologically active region and the results are plotted in Fig. 2.

Distribution of sugars on a paper chromatogram

A chromatogram of the crude eluate was prepared similarly to the one described above. After development for 24 hr. with the butanol-acetic acid-water mixture, the position of the sugars was determined by spraying the paper with aniline oxalate solution (Partridge, 1950). Seven spots were obtained: (i) pink, R_F 0·49; (ii) pink, R_F 0·22, probably arabinose; (iii) brown, R_F 0·19, probably glucose; (iv) brown R_F 0·16; (v) pink, R_F 0·13, possibly 2-ketogluconic acid; (vi) brown R_F 0·10, probably a polysaccharide; (vii) brown, R_F 0·05, probably a polysaccharide. The position of the arabinose, glucose and 2-ketogluconic acid spots agreed with those of authentic specimens run on the same chromatogram. The presence of pentoses was confirmed by a positive Militzer (1946) reaction on the crude stimulant solution. As the pentoses have greater R_F values than the hexoses, spot (i), which moved faster than any of the known pentoses, may have been a tetrose. However, erythrulose (Müller, Montigel & Reichstein, 1937), which as a ketose should be the most mobile of the three tetroses, had R_F 0·42 and gave a brown spot with aniline oxalate.

Chromatography on cellulose

A concentrate of the stimulant (77 mg.; R.A. 1000) from an ether-methanol extraction of the charcoal was dissolved in the upper layer (10 ml.) of the *n*-butanol-acetic acid-water mixture (Partridge, 1948) and a fraction (1 ml.) of the solution retained as a control. The remainder (9 ml. containing 69 mg. of crude stimulant of R.A. 1000) was brought on to a column (23 × 2 cm.) of powdered cellulose previously washed with the same solvent (150 ml.). The chromatogram was developed with more of the solvent and the eluate was collected in four 40 ml. fractions. The column was then extruded and the cellulose extracted with water (100 ml.). After removal of the solvent from the control and the various fractions at 0·1 mm. pressure and temperature $<40°$, the

residues were extracted separately with water (2 ml.) and the solutions tested for biological activity. The results are shown in Table 4.

Table 4. *Results of first chromatogram of the crude stimulant on cellulose columns*

Fraction	Total residue (mg.)	Water-soluble fraction (mg.)	Relative activity of water-soluble fraction
1	8·5	5·5	43,500
2	21·7	15·5	480
3	7·1	7·1	0
4	8·7	8·7	12·5
5 (from extruded column)	19·6	19·6	0
Material applied to column	69·0	60	1,280

A similar experiment was carried out using a $CHCl_3$-methanol extract of the charcoal (25·7 mg.; B.A. 7000) on powdered cellulose (10 g.) prewashed with the *n*-butanol-acetic acid mixture (200 ml.) as used in the previous experiment. The results obtained with 20 ml. fractions are shown in Table 5.

Table 5. *Results of second chromatogram of the crude stimulant on cellulose*

Fraction	Total residue	Volume of water used for extraction (ml.)	Water-soluble fraction (mg.)	Relative activity of water-soluble fraction
1	14·5	5	10·7	19
2	7·7	2	2·9	173,000
3	3·6	2	0·3	2,000
4	1·3	2	0·6	19
Material applied to column	25·7	—	—	7,000

DISCUSSION

It is clear that the stimulating properties of the linseed fluids are in a great measure and perhaps wholly due to the material which travels close to the solvent front in a *n*-butanol-acetic acid-water partition chromatogram, and which has an R_F value greater than 0·8 under the conditions specified. This material does not correspond with any of the carbohydrates detected on the chromatograms. The active region near the solvent front is a wide one, and it is possible that more than one active substance is concerned.

Substantial purification of the stimulant was achieved by the use of partition chromatography on cellulose columns, but in view of the resinous nature of the active material it was not possible to determine its purity. Other methods of purification have therefore been examined to ascertain whether concentrates could be prepared with comparable or even greater biological activity, and the results obtained in these investigations will be described in the succeeding paper of this series.

The relative stability of the stimulant in weakly acid solutions and its instability in alkaline solutions may well indicate the presence of an acidic or potential acidic (e.g. lactone) grouping. The reducing powers are also of great interest, but the significance of this property will only be clarified by further investigations on the purification and chemical structure of the stimulant.

SUMMARY

1. The stimulant required for the germination of *Orobanche minor* Sm. is unstable in alkaline solutions but moderately stable in weakly acid media.

2. Examination of a variety of solvents for the preliminary elution of the stimulant from charcoal has shown that a chloroform-methanol mixture is the most efficacious.

3. A method has been developed for the examination of the compound by paper-strip chromatography. With the *n*-butanol-acetic acid-water mixture, the factor travels very close to the solvent front and is quite distinct from the common carbohydrates. The method has been extended to partition chromatography on columns of cellulose, and concentrates with relative activity values of $1–2 \times 10^5$ have been obtained.

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An Electrophoretic Investigation of the Mixture of Oligonucleotides Formed by the Enzymic Degradation of Deoxyribonucleic Acid by Deoxyribonuclease

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The aim of the present work has been the investigation of the mixture of oligonucleotides formed by the action on deoxyribonucleic acid of the deoxyribonuclease isolated from pancreas by Kunitz (1948). Recent investigations of the nucleic acids concerning the analysis of the relative amounts of purine and pyrimidine bases set free on complete hydrolysis demonstrated the existence of different nucleic acids both in the deoxyribonucleic (DNA) and ribonucleic acid (RNA) series when isolated from different sources (Chargaff, Vischer, Doniger, Green & Misani, 1949; Vischer & Chargaff, 1948; Wyatt, 1950). That differences even exist between the DNA's of closely similar types of bacteria is also certain, due to the remarkable work of Avery, MacLeod & McCarty (1944) and Boivin, Delaunay, Vendrely & Lehoult (1945) on the transforming factors of pneumococci and *Escherichia coli* respectively.

The need for further investigation of these substances hardly requires stressing, but, for example, it may be mentioned that no satisfactory method for the isolation of the mononucleotides of the deoxy series yet exists. In addition little is known about the type of bonds joining these units into the chains of the nucleic acid molecule. Although it would certainly be preferable to investigate a nucleic acid specimen of known molecular homogeneity, some useful information may be obtained by studying hydrolysates of preparations which are actually mixtures of more than one nucleic acid. In fact certain preparations of DNA such as those of Hammarsten (1924) and Gulland, Jordan & Threlfall (1947) appear to be homogeneous when examined both by electrophoresis (Stenhagen & Teorell, 1939; Creeth, Jordan & Gulland, 1949) and by ultracentrifugation (Pedersen, 1940; Cecil & Ogston, 1948) and these results are supported by the recent work of Jungner, Jungner & Allgen (1949) on the dielectric behaviour of these substances. However, much further work is required before homogeneity can be regarded as established.

Despite this situation it seemed worth while investigating DNA as at present available both in order to develop suitable techniques, and because at least preliminary information bearing on molecular structure might be obtainable. Because investigations of intact nucleic acids have shown the DNA's as a class to have larger molecules than the RNA's, perhaps due to the milder conditions used for isolation, this investigation has been confined to thymus DNA.

As even slightly acid conditions lead to the destruction of mononucleotides by hydrolysis of the purine-deoxyribose bonds, and as the internucleotide bonds are inconveniently stable to alkali, the only possibility seemed to be to make use of enzymic hydrolysis. This approach was facilitated by the various studies which have already been made on the breakdown of DNA by deoxyribonuclease. Thus, in addition to specification of the optimum conditions for the activity of this enzyme, the work of Fischer, Böttger & Lehmann-Echternacht (1941), McCarty (1946) and Kunitz (1950) has made it clear that by its action DNA is converted into a mixture of oligonucleotides. Measurement of the average diffusion constant of this mixture indicated mean molar sizes corresponding to units of four (Fischer *et al.* 1941) or more (Kunitz, 1950) mononucleotides. Titration data show the liberation after complete enzymic hydrolysis of one acid group per four atoms P. Thus Fischer *et al.* (1941) have suggested that on the average the hydrolysate consists of tetranucleotides. Another important characteristic of the action of this enzyme is its failure to liberate inorganic P.

In the present work electrophoresis of the enzymic hydrolysate was carried out in jelly. After one such separation (Exp. 3) pieces of the jelly were cut out and an attempt was made to estimate the diffusion constants of the oligonucleotides contained in each. Another separation (Exp. 5) was conducted in a specially broad trough so that sufficient material could be obtained for quantitative analysis of the purine and pyrimidine bases present in those bands selected for analysis.

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METHODS

Materials. Deoxyribonucleic acid was prepared according to Hammarsten (1924) from calf thymus.

Deoxyribonuclease, crystalline material prepared according to Kunitz (1948) was purchased from the Worthington Biochemical Laboratory, Freehold, New Jersey, U.S.A.

The mononucleotides were commercial samples obtained from the National Biochemical Corporation, New York, with the exception of the sodium uridylate which was prepared in the laboratory. The diphosphopyridine nucleotide was kindly supplied by Dr J. B. Neilands.

Difco Bacto agar was set as a 1 % aqueous jelly and after cutting into small pieces was washed for 3 days in running tap water. The lumps of agar were then drained and stored in the cold room. This material will subsequently be referred to as washed 1 % agar. Washing the agar in this way served to remove most of the diffusible material and resulted in much stiffer jellies being formed at any concentration of agar.

Enzymic degradation of DNA. 500 ml. of a 0·2 % (w/v) solution of DNA was brought to pH 6·5 by the addition of 0·1 N-NaOH. $MgSO_4$ was added to make the solution $0{\cdot}3 \times 10^{-3}$ M with respect to Mg^{++}. Next 5 mg. enzyme were added and the solution was kept at 37° for 24 hr. The pH was maintained at 6·5 by the occasional addition of N-NaOH. No buffer could be used because of the subsequent electrophoresis. Finally, the digest, which no longer gave more than a very slight opalescence when acidified, was concentrated *in vacuo*.

Methods of electrophoresis. Electrophoresis was always conducted in agar jelly. Agar rather than silica jelly was used because it permits the passage of large molecules (Gordon, Keil, Šebesta, Knessl & Šorm, 1950) and it was desired to compare the mobility of the oligonucleotides with that of the original DNA. Agar is also convenient because solutions suitable for estimation in the Beckman quartz spectrophotometer can be made by melting pieces of the jelly with suitable volumes of water.

For all the separations except the preparative one (Exp. 5) a flat glass trough similar to that described by Consden, Gordon & Martin (1946) was used. Its internal dimensions were $85 \times 15 \times 0{\cdot}78$ cm. For the preparative experiment a similar trough having internal dimensions of $60 \times 60 \times 0{\cdot}78$ cm. was used. To ensure an adequate supply of buffer solution at the electrodes, branched tubes ending in four equally spaced glass taps were provided at each end of the jelly. In this work the jelly was never covered by a glass lid.

After the work had been in progress for some time it was found that ultraviolet-absorbing substances can be seen in the trough if a trace of a fluorescent substances is originally incorporated in the jelly which is then illuminated with ultraviolet light. This technique is also sufficiently sensitive to detect bands of protein in agar, if the proteins contain the usual proportions of tyrosine and tryptophan. The source of ultraviolet light was a Mineralight lamp giving maximum emission at 258 mμ. (Chargaff, Magasanik, Doniger & Vischer, 1949).

Owing to the range in which dissociation of the amino groups of the purine and pyrimidines present in the nucleotides occurs, useful mobility differences between such components are only to be expected at rather low pH values. On the other hand, care has to be exercised to avoid acidities at which hydrolysis of purine deoxyribose bonds can occur or at which there may be a possibility of precipitation. Because of these considerations most of the present work was carried out at pH 3·7. In addition a few experiments were carried out in acetate buffer at pH 4·4–4·6. As can be seen from Figs. 2 and 6 separation is not so good at pH 4·4 as at pH 3·7, but this had to be accepted in the case of the preparative experiment as the only buffers available which were suitable for the lower pH range also gave precipitates with mercury.

Examination of agar slab after electrophoresis. After examination with the ultraviolet lamp a row of suitably spaced rectangular cuts ($0{\cdot}8 \times 2{\cdot}0$ cm.) were made with a metal cutter along the length of the jelly. Next the pieces of jelly were lifted out of the cuts with a special spatula and placed in a series of test tubes. After the addition of 5 ml. water to each the jelly was melted. The solutions were then cooled and estimated by measurement of their extinction coefficients in the Beckman quartz spectrophotometer. Fortunately, between 248–310 mμ. no absorption due to fluorescein could be detected. Perhaps the greatest source of error in the values thus obtained results from losses of small pieces of jelly inadvertently allowed to remain in the trough.

Analysis for purines and pyrimidines. The solutions containing the oligonucleotides were evaporated to dryness *in vacuo*. The purines and pyrimidines were set free by hydrolysis with anhydrous formic acid as described by Wyatt (1950). For this purpose the oligonucleotides were dissolved in sufficient formic acid for the concentration of oligonucleotides to be approximately 20 mg./ml. The hydrolysis was carried out in 0·5 ml. portions in sealed tubes in an air bath at 175° for 30 min. After hydrolysis and centrifugation the black flakes were washed three times with a total of 6 ml. of warm formic acid. The combined supernatants were evaporated to dryness *in vacuo* and the excess formic acid removed by repeated evaporation after the addition of water.

The quantitative analysis of purine and pyrimidine bases was carried out by means of a chromatogram of Dowex 50 which was developed with 2 N-HCl as described by Cohn (1949). For this purpose each dry residue after the evaporation of formic acid was dissolved in 5 ml. 2 N-HCl and applied to the top of a Dowex column, height 90–100 mm., diameter 9 mm. Each chromatogram was developed and analysed as described by Cohn. Fractions containing the same base were quantitatively combined and the amount of base in each solution thus formed was determined by its light absorption in the Beckman quartz spectrophotometer. The calculations were carried out with constants which had been obtained previously by Reichard (1949*b*).

EXPERIMENTAL

Exp. 1. *Electrophoresis of DNA, adenylic, cytidylic, guanylic and uridylic acids at* pH 3·7

Washed 1 % agar (300 g.), 4 g. sodium hydroxide 21 g. citric acid and 4 mg. fluorescein were added to 700 ml. water and brought just to the boil. The solution was cooled to 60° and poured into the 15 cm. wide trough. The jelly thus formed was 0·78 cm. thick. After setting, a $5{\cdot}0 \times 0{\cdot}9$ cm. piece of jelly was cut out of the slab at a distance of 20 cm. from the cathode end of the trough. The gutter thus formed was filled with 3·5 ml. of a solution containing the same concentration of agar as that of the rest of

the jelly, 10 mg. each of DNA, adenylic, cytidylic and guanylic acids and sodium uridylate. No sodium citrate was added. Owing to the unavailability of the mononucleotides of the DNA series mononucleotides from RNA were used. It was assumed that compared with DNA-nucleotides no difference of mobility would be observable and that uridylic acid would move at practically the same rate as thymidylic acid. Impurities present in the mononucleotides no doubt account for the small extra peaks in Fig. 1.

The pH of the solution of the mononucleotides was so close to that of the rest of the jelly that no adjustment was necessary. Next the ends of the jelly were cut away and electrophoresis was carried out as described by Consden *et al.* (1946) and by Gordon *et al.* (1950). The solution at the electrodes was 0·1 M-sodium citrate at pH 3·7 First a potential gradient of 2·3 V./cm. was applied for 2 hr. and then 3·7 V./cm. for a further 15 hr. At the end of this period the jelly was examined as described above, light absorptions being noted at 240, 248, 262, 270 and 310 mμ. In this way the components responsible for each band were readily identified. The results thus obtained are shown in Fig. 1.

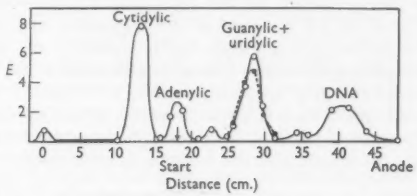

Fig. 1. Electrophoresis of DNA, adenylic, cytidylic, guanylic and uridylic acids at pH 3·7. ——, Light absorption at 262 mμ.; - - - -, light absorption at 248 mμ.

Exp. 2. *Electrophoresis of enzymic hydrolysate of DNA and DNA at* pH 3·7

The apparatus and jelly were the same as in Exp. 1. In this experiment, however, two 5·0 × 0·9 cm. gutters were cut out so that both DNA and a sample of the enzymic hydrolysate could migrate side by side along the trough. Into one was placed the usual agar solution containing 0·1 M-sodium citrate at pH 3·7 in which had been dissolved 21 mg. of DNA. During dissolution the temperature of this solution was never allowed to exceed 70°. The second was filled with a solution containing the oligonucleotides produced by the hydrolysis of 77 mg. DNA. In this and subsequent experiments where the enzymic hydrolysate was investigated the jelly in the inlay did not contain any buffer, the aim being always to keep the osmotic pressure of this part of the jelly as low as possible to prevent sweating. The pH was adjusted by the addition of N-citric acid. Electrophoresis was conducted for 4 hr. at 2·3 V./cm. and then for a further 19 hr. at 5·7 V./cm. Finally the jelly was examined as usual. By means of the ultraviolet lamp it was apparent that while most of the material had moved towards the anode, two extremely faint bands had moved towards the cathode. The results of the absorption measurements are shown in Fig. 2.

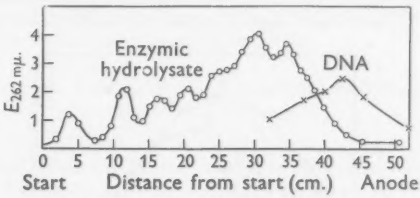

Fig. 2. Electrophoresis of enzymic hydrolysate of DNA and DNA at pH 3·7.

Exp. 3. *Electrophoresis of enzymic hydrolysate of DNA at* pH 3·7 *and diffusion measurements*

The apparatus and jelly were the same as in Exp. 1. The gutter was 11 × 0·9 cm. It was filled with 7·7 ml. of a solution at pH 3·7 containing agar and the oligonucleotides produced by the hydrolysis of 170 mg. of DNA. Electrophoresis was carried out as in the last experiment. Then the jelly was examined with the results shown in Fig. 3. As in Exp. 2, two faint bands were seen to have moved towards the cathode.

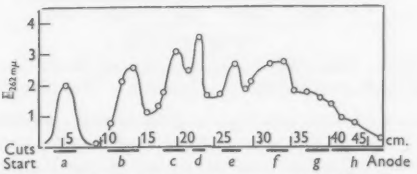

Fig. 3. Electrophoresis of enzymic hydrolysate of DNA at pH 3·7 (Exp. 3).

Using the information thus obtained eight sections were cut out from the jelly in the positions shown in Fig 3. After weighing and melting, each fraction was made up to 30 ml. by the addition of a hot solution of agar containing 750 g. washed agar/l. and 0·1 M-sodium citrate. These solutions were then transferred to flat-bottomed bottles of 4·0 cm. internal diameter. The concentration of agar was chosen partly because it gave jellies of adequate strength for the diffusion experiment, and partly because measurement of the thickness of the jelly in the trough at the end of the electrophoresis indicated that it had decreased to give a jelly of about this concentration. Thus, although different amounts of extra jelly were added for the resetting of each fraction, the final agar concentration in each bottle was nearly the same.

After complete setting, the fractions, together with one bottle containing only 0·1 M-sodium citrate agar and another which in addition contained approximately 7 mg. of cytidylic acid (ribose), were transferred to a thermostat air oven held at 27°. 1 hr. was allowed for temperature equilibration and then 30 ml. of the 0·1 M-citrate buffer was added to each bottle. The oven was equipped with stirrers which were now started so that the buffer solution

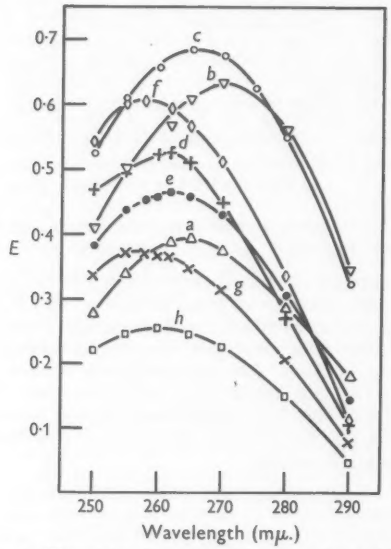

Fig. 4. Ultraviolet absorption of fractions obtained by electrophoresis of enzymic hydrolysate of DNA (Exp. 3).

over each jelly was kept in motion. After a further 1, 2, 3, 4 and 5 hr. respectively small portions of the solutions were examined in the Beckman spectrophotometer and quickly returned to the bottles. Finally, in order to ascertain the concentration of ultraviolet-absorbing material which must have been present in the jelly at the start of the experiment, each bottle was emptied into a beaker, the jelly was melted and made up to 400 ml. with 0·1 M-sodium citrate. The light absorption of these solutions was measured at a number of wavelengths; the curves thus obtained being shown in Fig. 4. As a control experiment approximately similar amounts (7–10 mg.) of adenylic, guanylic, cytidylic and uridylic acids, diphosphopyridine nucleotide, DNA and unfractionated enzymic hydrolysate were set in agar in the bottles and allowed to diffuse into 0·1 M-citrate buffer. Measurements were again made after 1, 2, 3, 4 and 5 hr. The results of all these diffusion measurements have been calculated in the manner suggested by Friedman & Kraemer (1930) and are shown in Table 1.

Table 1. *Diffusion constants of fractions obtained by electrophoresis of enzymic hydrolysate of DNA (Exp. 3) compared with those of mononucleotides, coenzyme I and intact DNA*

Substance	Diffusion constant (sq.cm./sec. × 10^{-6})
DNA	0·25
Coenzyme I	4·9
Adenylic acid	6·2
Guanylic acid	6·0
Cytidylic acid	6·0
Uridylic acid	6·8
Enzymic hydrolysate	3·8
Fraction *a*	4·3
Fraction *b*	3·7
Fraction *c*	4·5
Fraction *d*	4·7
Fraction *e*	3·4
Fraction *f*	3·5
Fraction *g*	3·0
Fraction *h*	3·9

Exp. 4. *The electrophoresis of the enzymic hydrolysate at* pH 4·6

The apparatus and jelly were the same as in Exp. 1 except that 0·2 M-sodium acetate at pH 4·6 replaced the citrate buffer in the jelly and 0·3 M-acetate buffer at pH 4·6 was used at the electrodes. The use of a more concentrated buffer solution at the electrodes was found to be advisable at this pH to prevent the jelly becoming swollen with liquid near the anode. Unless this was done the area of swollen jelly extended during the electrophoresis and sometimes interfered with the location of the substances.

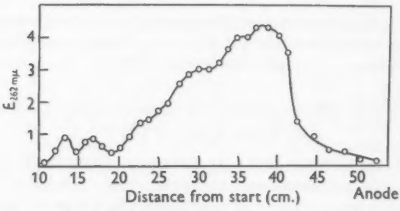

Fig. 5. The electrophoresis of the enzymic hydrolysate of DNA at pH 4·6.

Next a gutter was cut out and a sample of the enzymic hydrolysate in agar at pH 4·6 was added just as in Exp. 2. Electrophoresis was then conducted for 8·5 hr. at 2·3 V./cm. and then for 13·5 hr. at 5·7 V./cm. Finally the jelly was examined as usual, the curve obtained being shown in Fig. 5.

Exp. 5. *Large scale electrophoresis of the enzymic hydrolysate at* pH 4·4

The large trough was filled with 2·8 l. of solution made up as in Exp. 1 except that 0·2 M-sodium acetate replaced the citrate. After spaces had been

made for the electrodes at the ends of the trough a gutter 58 × 0·9 cm. was cut out at a distance of 6 cm. from the cathode edge of the jelly and parallel with it. This was then filled with 37 ml. of the usual agar solution containing the oligonucleotides produced by the hydrolysis of 1 g. of DNA. Electrophoresis was conducted for 5 hr. at 4·1 V./cm. and then for a further 16 hr. at 6·7 V./cm. Probably because the concentration of oligonucleotides and salt in the inlay was too high, considerable 'sweating' occurred in this region. As much as possible of this free liquid was removed. After the electrophoresis the jelly was examined with the results shown in Fig. 6. By means of this information and making use of the ultraviolet lamp to ascertain that the bands had not become curved, pieces of jelly were cut out as shown in Fig. 6.

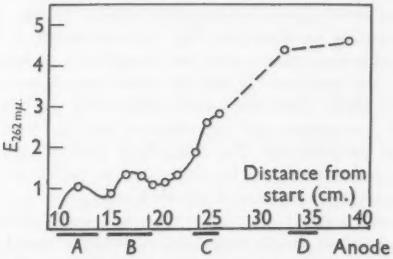

Fig. 6. Large scale electrophoresis of enzymic hydrolysate of DNA at pH 4·4 (Exp. 5).

Each section of agar was allowed to stand in 500 ml. of water for 24 hr. In this way the greater part of the salts and oligonucleotides diffused out. The solution was filtered off and extraction was repeated with a further 500 ml. of water. The combined solutions were then evaporated *in vacuo* to a volume of about 10 ml.

In order to free the oligonucleotides from salts and from almost all the agar they were precipitated with Hg^{++}, a method which has been applied by Reichard (1949*a*) to the preparation of ribomononucleotides. Following this method after adjusting the pH to 2·5 with 5 N-nitric acid, mercuric nitrate was added. The resulting precipitates were allowed to stand for 48 hr., washed and freed from mercury by treatment with hydrogen sulphide. The completeness of precipitation with mercury was checked by measuring the light absorption at 262 and 310 mμ. of the supernatant and washings. No indication of the presence of free purines or pyrimidines was obtained. Finally the purine and pyrimidine contents of the solutions were estimated by the method described above, and the results are given in Table 2, together with an analysis of the original DNA.

Exp. 6. *Electrophoresis of the enzymic hydrolysate at* pH 7 *in a strong jelly*

This experiment was carried out similarly to the electrophoresis of the enzymic hydrolysate described in Exp. 2 except that the jelly was made by melting 1 % washed agar without the addition of water. 0·1 M-sodium phosphate buffer at pH 7 was used in the jelly and at the electrodes. The results obtained are shown in Fig. 7.

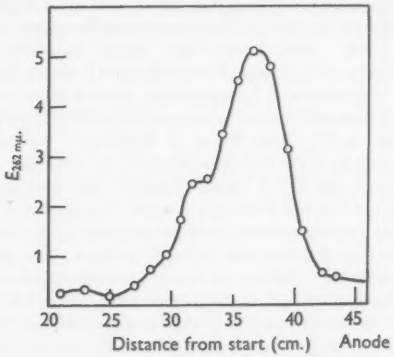

Fig. 7. Electrophoresis of enzymic hydrolysate of DNA at pH 7 in a strong jelly.

DISCUSSION

Little difficulty was experienced in adapting for the present purpose the experimental conditions used previously for the electrophoresis of proteins

Table 2. *Amounts of purine and pyrimidine bases found after hydrolysis and chromatography of fractions obtained by electrophoresis of enzymic hydrolysate of DNA* (*Exp.* 5)

(*R* = molar ratio of cytosine to the other bases.)

	Thymine		Cytosine		Guanine		Adenine	
	mg.	*R*	mg.	*R*	mg.	*R*	mg.	*R*
DNA	0·77	1·22	0·55	1·00	0·69	0·92	0·75	1·12
Fraction *A*	0·21	0·16	1·15	1·00	0·10	0·06	0·93	0·66
Fraction *B*	0·61	0·44	1·21	1·00	0·16	0·10	0·19	0·13
Fraction *C*	1·04	0·55	1·64	1·00	0·62	0·28	1·18	0·66
Fraction *D*	1·50	1·82	0·75	1·00	0·70	0·68	1·01	0·99

(Gordon *et al.* 1950). However, an initial complication was encountered when an attempt was made to investigate intact DNA at pH 5. Perhaps not surprisingly, in view of the strong tendency of DNA to form jellies, part of the ultraviolet-absorbing material originally set in the inlay scarcely moved from this position; another part of the DNA, however, moved towards the anode in the manner expected. It would be of interest to ascertain whether any real fractionation of DNA can be achieved in this way. Presumably because DNA cannot form jellies as easily at pH 3·7 this type of behaviour was not observed in any of the later experiments. However, even at this pH value the DNA did not give as regular a band as those obtained from substances of lower molecular weight. Thus observation with the ultraviolet lamp of DNA which had moved a considerable distance through the jelly, sometimes revealed a single band with irregular front and back. Such bands also caused considerable reduction in thickness of the jelly, and it seems likely that the observed irregularities were due to some such cause rather than to any possible inhomogeneity of the DNA. This type of behaviour probably occurred in Exp. 2 as is evidenced by the very flat shape of the DNA band. Despite this bad shape there is no doubt that only a small proportion of the fastest moving molecules of the enzymic hydrolysate moved as fast as did the DNA itself. The most probable explanation of this behaviour is the compactness of the intact DNA molecule. Thus it seems that this factor must more than counterbalance those likely to tell in the opposite sense. These latter presumably must include the extra negative charges introduced on enzymic hydrolysis and any tendency of the DNA to be held back by the jelly either by absorption or by a molecular sieve effect (Synge & Tiselius, 1950). That these two latter effects are negligible in agar at least for the enzymic hydrolysate was shown in Exp. 6 in which a very strong jelly was used.

The main result of the electrophoresis of the enzymic hydrolysates in their division into a number of distinct bands. The variation of the relative mobilities of some of these with pH is immediately apparent if the results obtained at pH 3·7 and 4·6 are compared. Thus at the higher pH the two slower moving bands are obviously less well resolved from the main bulk of the absorbing material. Even if all these bands finally prove to contain more than one molecular species the possibility of analysing the mixture produced by the enzyme begins to emerge. Another deduction which can be made directly from the results of the electrophoreses is that only very little guanine or adenine, if any, is split off as such by the action of the enzyme. This follows from the observation in Exp. 2 of two extremely faint bands moving towards the cathode. In Exp. 3 two similar bands were observed; judged by their appearance with the ultraviolet lamp they were only a little stronger. A more important difference between the two enzymic hydrolysates is, however, immediately apparent on comparison of Figs. 2 and 3, i.e. the much greater heights of the slower moving bands in the latter. Just possibly these differences may be due to the fact that the second hydrolysate was made with a different batch of deoxyribonuclease; the enzyme was not subjected to any purification after purchase. It seems more likely, however, that the differences may be due simply to inadequate control of the conditions during the hydrolysis.

Owing to the very incomplete separation of all but the two slowest moving bands the attempt to ascertain the bases present in each was restricted to these two. Cuts *C* and *D* in Fig. 5 were made for control purposes and to gain general information about the type of separations taking place. Unfortunately as shown in Fig. 5 even bands *A* and *B* in this experiment were not completely separated from one another. If this be taken into account it seems likely that the main component present in band *A* contains only cytosine and adenine in equimolar proportions. The analytical figures actually obtained could thus be explained as being due to overlapping with band *B* which was found to contain thymine and cytosine in the molecular ratio of about 1:2. Another simple explanation would be that band *A* contains at least two oligonucleotides, one containing cytosine and adenine in the molecular ratio 2:1, the other adenine and thymine in the ratio 1:1. However, this hypothesis seems unlikely as a dinucleotide of adenine and thymine containing two phosphoric acid groups would be expected to have a rather higher electrophoretic mobility than that of band *A*. The mobilities of bands *A* and *B* make it extremely unlikely that these bands consist of mixtures of mononucleotides. Only thymidylic acid could be present in band *B*, but the additional presence of cytosine in this band makes it much more likely that both are parts of an oligonucleotide. The presence of guanine in both these bands and adenine in band *B* can no doubt again be explained as being due to contamination with contiguous bands.

The diffusion measurements referred to above are also relevant to the question as to whether bands *A* and *B* contain mainly di- and tri-nucleotides or on the other hand substances of much higher molecular weight. Owing to the presence of agar these measurements could not be made in the usual diffusion cell and cannot therefore be compared with those obtained by Kunitz (1950) on the unfractionated hydrolysate of DNA. As the method employed here was originally worked out for non-electrolytes no claim is made that the figures obtained (Table 1) are of absolute significance. In addition the rather high blank values given by agar itself are a source of

inaccuracy. Despite these facts two conclusions seem to be legitimate. They are that there is no great difference in respect to the diffusion constants of the electrophoretically slower and faster moving components of the enzymic hydrolysate and that the diffusion constants of bands *A* and *B* are not inconsistent with those that might be expected for di- and tri-nucleotides. As mixtures containing dinucleotides have been found to be separable by means of paper chromatography (Hummel & Lindberg, 1949) it would seem worth while to test out the suitability of this technique for the further analysis of the fractions obtainable by electrophoresis.

SUMMARY

1. Deoxyribonucleic acid has been hydrolysed by means of the deoxyribonuclease of ox pancreas.

2. The mixture of oligonucleotides thus formed has been subjected to electrophoresis in agar jelly.

3. The incorporation of a trace of fluorescein in the jelly has made possible the visualization of bands of ultraviolet-absorbing material.

4. After electrophoresis appropriate sections have been cut out of the jelly and the diffusion constants of the materials contained in each has been estimated.

5. The ratios of the purine and pyrimidine bases present in such cuts have been estimated after hydrolysis and separation on an ion-exchange chromatogram.

6. The evidence thus obtained suggests that the main components of the two slowest moving bands are respectively dinucleotide(s) of adenine and cytosine and trinucleotide(s) containing one residue of thymine and two residues of cytosine.

Sincere thanks are due to Mrs Lisa Vestermark for technical assistance.

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Improved Nomograms for Manometer Constants

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Biochemical Laboratory, University of Cambridge

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Dixon (1945) published a set of nomograms for the rapid determination of the constants of Warburg and Barcroft manometers. They have proved useful in practice and have saved much time and calculation, but could only be used for one, or at the most two, fixed temperatures. For work at other temperatures it was necessary either to construct new nomograms or to determine the constants by calculation.

Burris (1949) later devised a nomogram for Warburg manometers which could be used for any temperature up to 45°, but it was inconveniently complicated and required three separate settings of the straight edge (in addition to the calculation of v_G from V and v_F) to determine each constant. No such nomogram has been produced for Barcroft (differential) manometers.

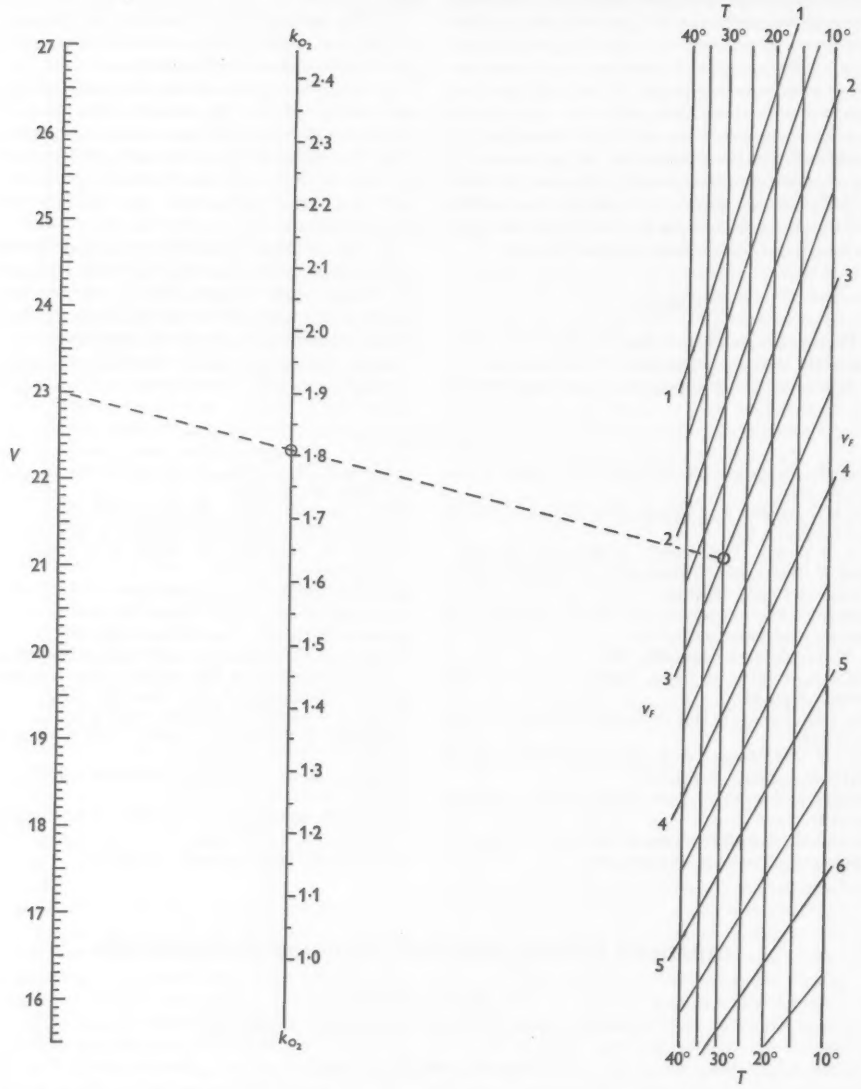

Fig. 1. k_{O_2} for Warburg manometers. Stretch a fine black thread across the nomogram so as to connect the given value of V with the point of intersection of the lines for v_F and T, and read off k_{O_2}. Intermediate values can readily be estimated visually. The example indicated shows that a manometer with a 23 ml. vessel containing 3 ml. of liquid has a k_{O_2} of 1·81 at 30°.

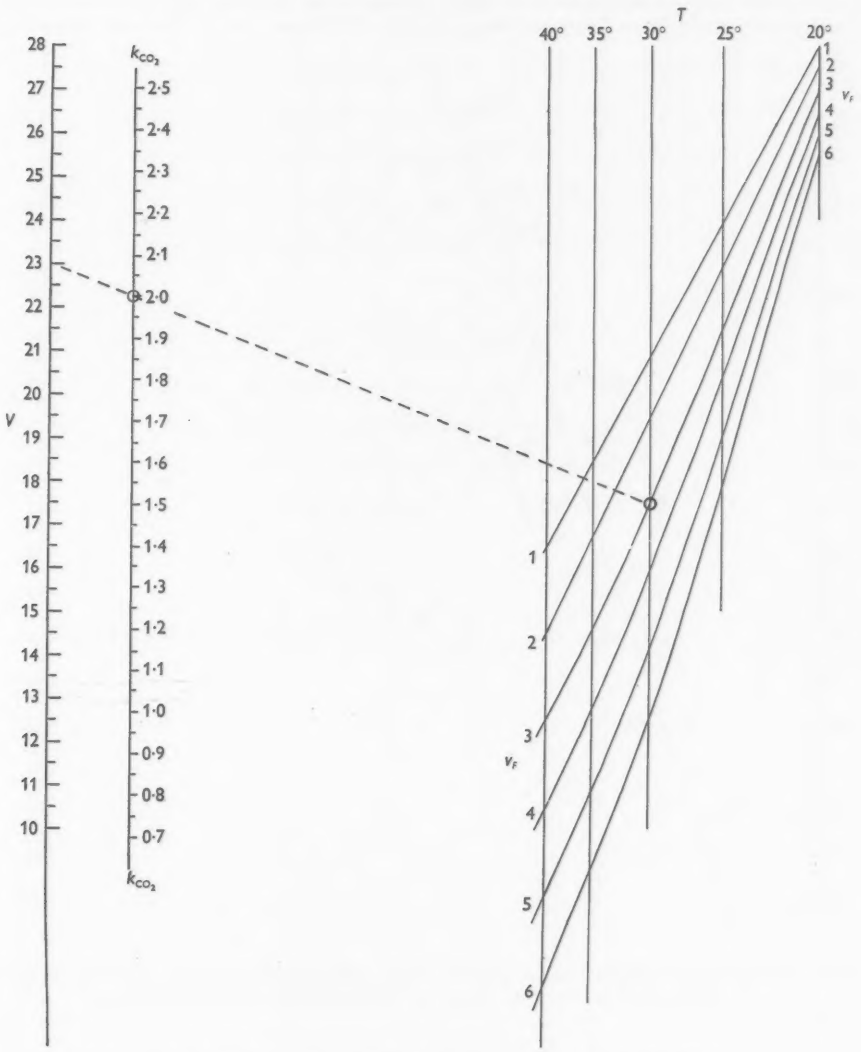

Fig. 2. k_{CO_2} for Warburg manometers. To be used in the same way as Fig. 1.

It has now been possible, by making use of the principle of the grid nomogram, to construct nomograms for both Warburg and Barcroft manometers which can be used for any temperature in the usual range, and yet are practically as simple and rapid in remaining scale (or scales) then moves as the temperature alters, thus forming a grid.

It is assumed that Brodie solution ($P_0 = 10{,}000$ mm.) is used in the Warburg manometers and paraffin oil ($P_0 = 13{,}100$ mm.) in the Barcroft manometers, as

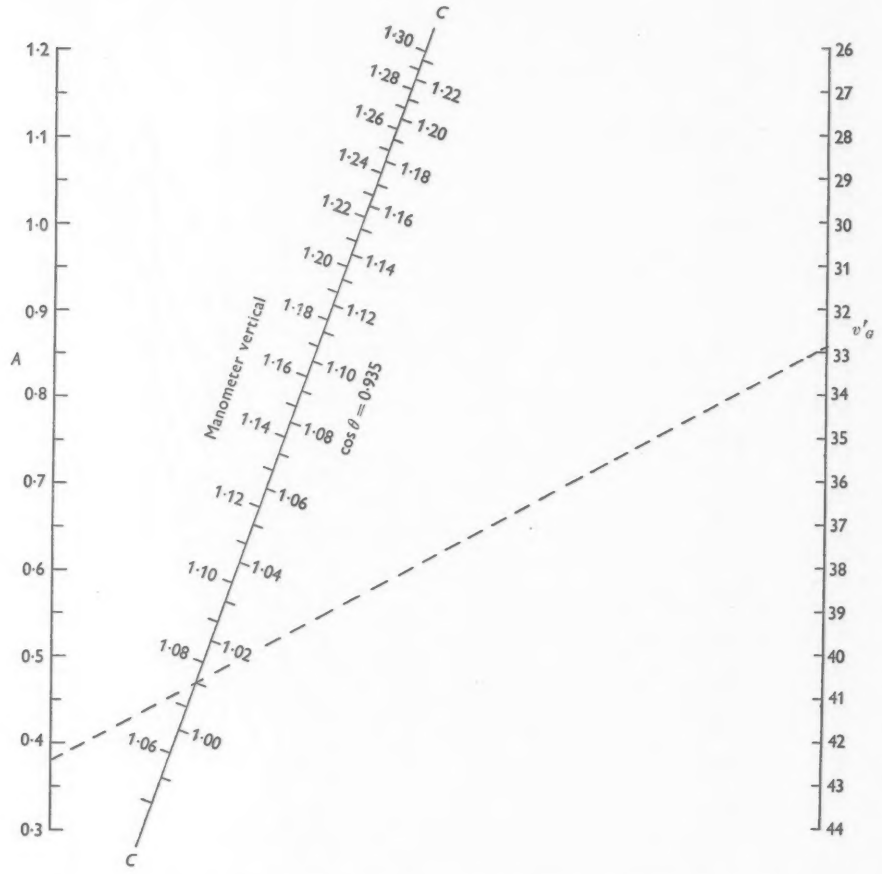

Fig. 3. C for Barcroft manometers. Connect the given value of A, the area of cross section of the manometer tube in sq.mm., with v'_G, the volume of the gas phase in the compensating vessel in ml.

use as those originally published. Figs. 1 and 2 give k_{O_2} and k_{CO_2} respectively for Warburg manometers and Figs. 3–5 give constants for Barcroft manometers.

For constructional purposes they may be each regarded as consisting of a large number of superimposed nomograms for different temperatures, in which the positions and magnifications of two of the scales are arbitrarily fixed at constant values. The this is the usual practice. These nomograms do not apply to manometers containing heavy liquids, e.g. Clerici solution. The volumes are given for convenience in ml. and not in μl. It should be noted that V always refers to the total volume of the vessel, as determined by calibration with mercury; the preliminary calculation of v_g is therefore rendered unnecessary.

For Barcroft manometers $k = CD$, C being given by Fig. 3, and D for O_2 by Fig. 4 and for CO_2 by Fig. 5. In Fig 3 scales are given for vertical as well as for tilted manometers, as it is now becoming common practice to use Barcroft manometers with vertical shakers.

It will be noted that at about 18° k_{CO_2} becomes independent of v_F. This is because at this temperature 1 ml. of water contains as much CO_2 as 1 ml. of the gas phase at any partial pressure of CO_2.

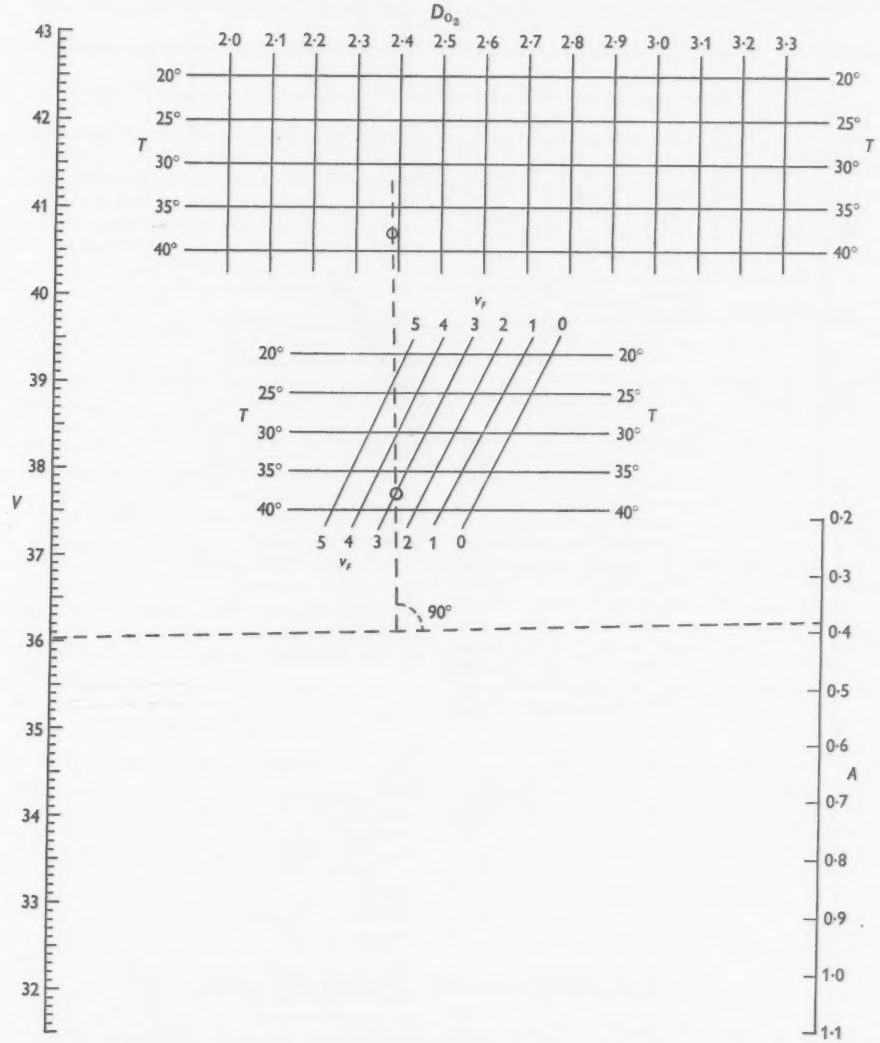

Fig. 4. D_{O_2} for Barcroft manometers. Place a straight edge to connect V with A. On this place a 90° set square and slide it along until it passes through the point of intersection of v_F and T on the central grid. D is then read off on the upper grid at the correct level of T. The example shows that a manometer with a 36·03 ml. vessel containing 3 ml. of liquid and a 0·38 sq.mm. tube has a D of 2·385 at 38°. C for this manometer = 1·01 (from Fig. 3) so that $k_{O_2} = 2·41$.

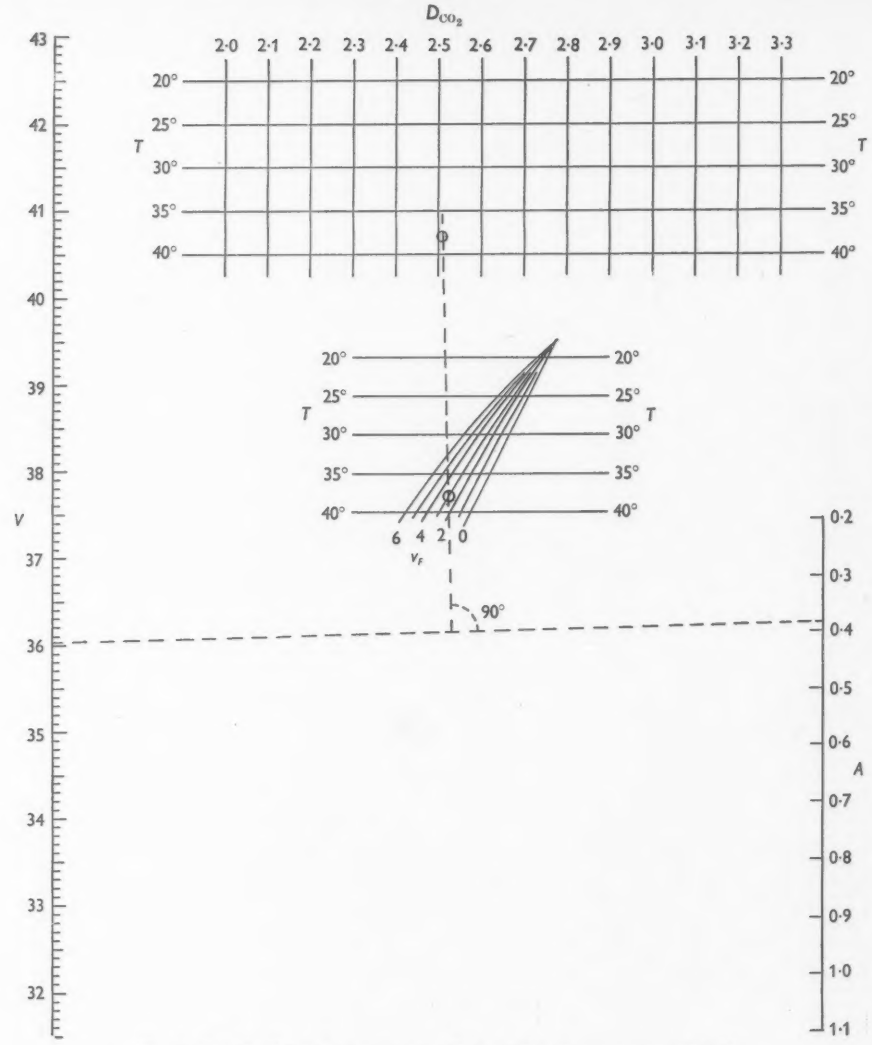

Fig. 5. D_{CO_2} for Barcroft manometers. To be used in the same way as Fig. 4.

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Recognition and Estimation of 5-Methylcytosine in Nucleic Acids

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The pyrimidine 5-methylcytosine was first reported as a constituent of a nucleic acid by Johnson & Coghill (1925), who prepared from hydrolysed tuberculinic acid a crystalline picrate which was identified as 5-methylcytosine picrate by microscopical examination. However, when Vischer, Zamenhof & Chargaff (1949) examined nucleic acid from avian tubercle bacilli by chromatography, they could not detect this substance. Nevertheless, Hotchkiss (1948) noted on paper chromatograms of hydrolysed thymus nucleic acid a small spot whose ultraviolet spectrum and chromatographic behaviour led him to suggest that it might be 5-methylcytosine.

As already reported in a preliminary note (Wyatt, 1950), I have found by a simple chromatographic method that 5-methylcytosine seems to occur in constant amounts in deoxypentose nucleic acids (DNA) from animals and from at least one higher plant, but has not so far been found in DNA from microbial sources. The isolation, partial characterization and estimation of this substance will now be described. Quantitative results will be presented along with the method and results of estimating the other pyrimidine components of some DNA's in the succeeding paper (Wyatt, 1951).

EXPERIMENTAL

Isolation of the new substance

The purines and pyrimidines set free by hydrolysis of DNA with formic acid (175° for 30 min.) were separated on paper chromatograms run with aqueous 65% (v/v) *iso*propanol 2N in respect of HCl as described by Wyatt (1951), and their positions detected by the photographic technique of Markham & Smith (1949). In preliminary experiments with DNA from ox spleen and from herring sperm, it was noted that in addition to guanine, adenine, cytosine, thymine and sometimes a trace of uracil, another substance formed a faint spot which moved slightly more rapidly on the chromatograms than cytosine. Tests had shown that the substance was not cytosine deoxyriboside, since its concentration was not appreciably diminished by increasing the hydrolysis time, and was not an aromatic amino-acid, since it gave no colour with ninhydrin. The isolation of a sample was undertaken as follows.

40 g. of crude herring-sperm DNA were freed of purines by methanolysis with HCl (Levene & Bass, 1931, p. 110). The filtrate from the precipitated purine hydrochlorides was evaporated to dryness, dissolved in 6N-HCl, and heated at 125° for 2 hr. to complete liberation of the pyrimidines. The hydrolysate was clarified with activated charcoal, evaporated to dryness under diminished pressure, redissolved in water and again evaporated. The tarry residue was taken up in water and freed of H_3PO_4 by neutralization with $Ba(OH)_2$ and filtration. After evaporation to a small volume, a crystalline precipitate separated out, consisting mainly of thymine; after further reduction in volume a precipitate containing cytosine, thymine and some of the new substance formed. The filtrate was made alkaline with NaOH and the remaining pyrimidines were precipitated with $AgNO_3$. After removal of Ag^+ with HCl this fraction was found to contain the new substance along with some thymine, uracil, adenine and much cytosine.

This concentrate was fractionated by paper chromatography. The solution was applied as a band across the top of sheets of Whatman no. 3 filter paper, which were run as chromatograms in *iso*propanol-aqueous HCl. In order to shorten exposure time, because of the thickness of Whatman no. 3 paper, Ilford Document Paper, no. 60, was used for printing these chromatograms instead of Reflex Document Paper, no. 50. As the chromatograms were overloaded (about 10 mg. of each substance can be separated on an 18 × 22 in. sheet of Whatman no. 3 paper), resolution was poor, but the bands containing the new substance were eluted and the eluate chromatographed in *n*-butanol-aqueous NH_3. The eluate this time contained only one ultraviolet-absorbing substance, and on evaporation deposited microscopic crystals.

Comparison with 5-methylcytosine

On paper chromatograms the new substance moved slightly more rapidly than cytosine in all the solvents tested, which included *iso*propanol-aqueous HCl, *n*-butanol-water, *n*-butanol-aqueous formic acid or NH_3, and *iso*amyl alcohol-aqueous NH_3. The position relative to cytosine was similar regardless of pH, but the separation from cytosine increased the higher the alcohol used. This strongly suggested a compound bearing ionizable groupings identical with those of cytosine, but differing by an additional alkyl group, and 5-methylcytosine seemed a likely possibility.

An authentic sample of 5-methylcytosine was not at the time available, but some synthetic 2-amino-4-hydroxy-5-methylpyrimidine (5-methyl*iso*cytosine) was kindly given by Prof. A. R. Todd, F.R.S. This had absorption spectra different from those of the unknown, and on chromatograms moved slightly faster.

If the unknown were 5-methylcytosine, it would be converted to thymine by deamination with HNO_2. Accordingly, 0·5 mg. of the substance in 1 ml. water was mixed with 2M-$Ba(NO_2)_2$ (2 ml.) and glacial acetic acid (0·5 ml.). After 6 hr. at room temperature, the excess acetic acid was removed by evaporation, and Ba^{++} with H_2SO_4. The filtrate from $BaSO_4$ was evaporated to dryness and dissolved in 0·1 ml.

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of water, and spots were applied to paper for chromatography. A portion of the synthetic 5-methyl*iso*cytosine was treated in the same way. Both were found to have been converted quantitatively to a substance identical with thymine in its chromatographic movement (Fig. 1) and ultraviolet absorption spectrum.

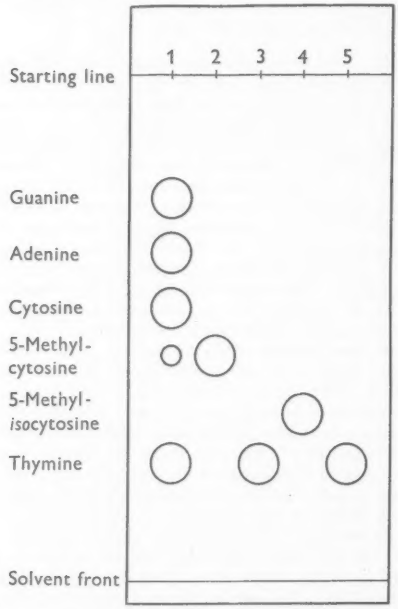

Fig. 1. Diagram of a paper chromatogram run in *iso*propanol-aqueous 2N-HCl. Solutions applied at positions 1–5 were: (1) hydrolysate of herring-sperm deoxypentose nucleic acid (DNA); (2) supposed 5-methylcytosine isolated from DNA; (3) product of deamination of (2); (4) synthetic 5-methyl*iso*cytosine; (5) product of deamination of (4).

There are only three substances which would yield thymine on deamination: 5-methylcytosine, 5-methyl*iso*cytosine and 2:4-diamino-5-methylpyrimidine. The unknown had been found different from the second of these, and was most unlikely to be the third, since the possession of an additional amino group would cause slower movement in acid and more rapid movement in ammoniacal solvents on the chromatograms. This made it reasonably certain that the new substance was 5-methylcytosine.

After these experiments had been completed, a sample of 5-methylcytosine synthesized by the method of Wheeler & Johnson (1904) was obtained through the kindness of Dr A. Pircio, Fordham University, New York. This proved to be identical with the natural substance in its movement in both acid and ammoniacal chromatographic solvents and in its ultraviolet absorption spectra in neutral, acid and alkaline solution (Fig. 2). These spectra are also identical with those recently published by Hitchings, Elion, Falco & Russell (1949) for 5-methylcytosine prepared by a new synthesis.

Since the absorption curves and chromatographic behaviour provide evidence for the identity of the natural and synthetic substances, isolation of a sample sufficient for accurate elementary analysis has not been attempted. Owing to the low capacity of paper chromatograms, only a small portion of the concentrate obtained by precipitation had been fractionated by chromatography. Separation on starch columns was also tried, but their capacity proved

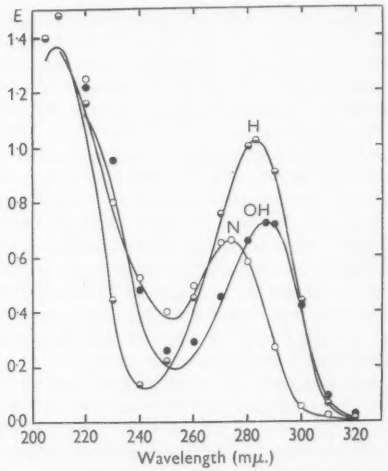

Fig. 2. Ultraviolet absorption spectra of 5-methylcytosine in 0·1N-HCl (◒), in 0·1N-NaOH (●) and at pH 7·0 (○). The curves represent the absorption of synthetic 5-methylcytosine (0·013 mg./ml.), and the points that of natural 5-methylcytosine eluted from chromatogram spots and read against paper blanks.

little greater than that of filter paper. Fractional crystallization proved useless for separation of cytosine and 5-methylcytosine. If it were desired to isolate a greater amount of natural 5-methylcytosine, a better starting material would be DNA from wheat germ, which contains one-third as much 5-methylcytosine as cytosine. The final fractionation might be accomplished by counter-current distribution or on ion-exchange columns.

Ultraviolet absorption characteristics of 5-methylcytosine

For determination of its ultraviolet absorption characteristics, some synthetic 5-methylcytosine was purified through the picrate, recrystallized from methanol and from water, and dried at 110° for weighing. Standard solutions were made up in water (taken to pH 7·0 with a little NaOH), 0·1N-HCl and 0·1N-NaOH. Molecular extinction coefficients at maxima and minima are given in Table 1. In acid solution there is an additional maximum at about 211 mμ. The characteristic shifts in acid and alkali correspond closely to those of cytosine, the wavelengths of absorption maxima being in each case longer by 6–8 mμ., and those of the minima by 3–4 mμ. The shift from the 'acid' to the 'neutral' spectrum takes place at a pH just below 7, and that from

the 'neutral' to the 'alkaline' at about pH 12, so that the curve for pH 7 (Fig. 2) and that for pH 11 (Hitchings *et al.* 1949) are almost identical.

Table 1. *Ultraviolet absorption characteristics of 5-methylcytosine*

(ϵ is molecular extinction coefficient.)

	Maxima		Minima	
Solvent	Wavelength (mμ.)	ϵ	Wavelength (mμ.)	ϵ
Water	274	6260	252	3680
0·1 N-HCl	283	9810	242	950
0·1 N-NaOH	287	6870	253	1670

Method of estimation

The amount of methylcytosine in animal nucleic acids is so small that estimation by elution of the spot on chromatograms of unfractionated hydrolysate is inaccurate, especially if traces of absorbing impurities are present. A procedure has therefore been used which is in effect two-dimensional chromatography, the substances being concentrated after running in the first dimension. Hydrolysate of 6–8 mg. of DNA is applied in a band 25 cm. long across the top of a sheet of paper, which is then run in *iso*propanol-aqueous-HCl, dried and printed. A transverse band containing all of both cytosine and methylcytosine is cut from the chromatogram; one end of this band is cut to a point, and the other end is placed in a trough containing water in a chromatogram tank. The pyrimidines move with the water front and are eluted quantitatively in the first 0·5 ml. to drip from the bottom of the paper strip, which is collected in a small tube. This is then evaporated to dryness and redissolved in 0·1 N-HCl (0·04 ml.). Measured volumes are placed as spots on filter paper and run in *n*-butanol-aqueous NH_3, in which cytosine and methylcytosine are well separated from each other and from impurities extracted from the paper of the first chromatogram. The separated spots are eluted and their pyrimidine contents estimated on the Beckman spectrophotometer; the amount of cytosine serves as a standard for referring the amount of methylcytosine to the whole nucleic acid. In this way methylcytosine can be estimated when it comprises as little as 0·1 % of the original nucleic acid.

Distribution of 5-methylcytosine

The various animal DNA's so far analysed (including mammals, a fish, an insect and an echinoderm) all contain methylcytosine, in amounts characteristic of the species source, and varying from 0·008 to 0·075 mol./4 mol. of nucleotide. The only DNA from a higher plant analysed, that of wheat germ, contains much more, 0·23 mol./4 mol. of nucleotide (Wyatt, 1951). None could be detected in DNA from the following microbial sources: human and bovine tubercle bacilli, *Escherichia coli*, *Esch. coli* bacteriophages T_2 and T_5, and the virus of the polyhedral disease of gipsy moth larvae; none or extremely little is present in DNA from baker's yeast. Nor could any be found in the ribonucleic acids of yeast and of turnip yellow-mosaic virus.

DISCUSSION

In the present state of knowledge as to the structure and function of nucleic acids nothing can be said as to the possible function of 5-methylcytosine. The amounts in which it occurs, however, varying with the source but constant from a given source, suggest that it is an essential constituent of certain DNA's, and no accident of enzyme action. The demonstration that these nucleic acids do contain this pyrimidine in addition to their well known components extends the evidence of recently published analyses that their structure is a matter of considerable complexity and worthy of much further investigation. In particular, it would be interesting to know whether the observation that methylcytosine is present in higher organisms but lacking in micro-organisms holds generally, and if so, at what point in the evolutionary scale it first appears.

It is ironical that the nucleic acid of tubercle bacilli, from which Johnson & Coghill (1925) originally reported 5-methylcytosine, should be one of those which do not contain it. As a clue to the source of their error, it is interesting to note that Vischer *et al.* (1949) examined by chromatography a specimen of methylcytosine obtained from Prof. T. B. Johnson and found it to contain 42 % of cytosine, so that the crystalline '5-methylcytosine picrate' with which the picrate from tuberculinic acid was compared may well have been chiefly that of cytosine.

SUMMARY

1. An aminopyrimidine which on deamination gives rise to thymine has been discovered as a new constituent of some deoxypentose nucleic acids (DNA). The ultraviolet absorption spectra and chromatographic behaviour of this substance are identical with those of synthetic 5-methylcytosine, and the tentative conclusion is drawn that it is the latter.

2. By a simple chromatographic technique, this pyrimidine can be estimated when occurring in amounts as small as 0·1 % of a nucleic acid.

3. Ultraviolet absorption spectra of 5-methylcytosine have been determined.

4. The new pyrimidine occurs in amounts characteristic of the source in all of the animal DNA's and the one plant DNA analysed, but is apparently lacking from DNA of bacteria and viruses, and from ribonucleic acids.

I wish to thank Dr J. D. Smith for information on his analyses of nucleic acid from *Esch. coli* and its bacteriophages, Dr R. Markham for helpful advice and discussion, and Dr K. M. Smith, F.R.S., for providing facilities for the work. The work was carried out while I was on transfer of research from the Forest Insect Investigations Unit, Division of Entomology, Department of Agriculture, Canada, to the Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge. This paper is Contribution No. 2701, Division of Entomology, Science Service, Department of Agriculture, Ottawa, Canada.

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The Purine and Pyrimidine Composition of Deoxypentose Nucleic Acids

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Although the composition of nucleic acids has been subject to frequent investigation since these compounds were first isolated by Miescher in the last century, it is only through the application of techniques developed in the past few years that their accurate quantitative analysis has become possible. Early analyses by macrochemical methods (Steudel, 1906; Levene & Mandel, 1908) indicated that nucleic acids contained two purine and two pyrimidine bases in approximately equimolecular proportions. This gave rise to the tetranucleotide hypothesis, upheld by Levene & Bass (1931) and widely accepted, according to which the unit of nucleic acid structure is a group of four nucleotides comprising one of each of the four bases. Recent micro-analyses, however, by such methods as filter-paper chromatography and ultraviolet spectrophotometry, have shown that the composition of nucleic acids is not consistent with any such simplified theory, a conclusion which is in line with biological evidence of their intimate function in cell physiology and of the specific activities of certain of them, e.g. in inducing mutations in bacteria (Avery, MacLeod & McCarty, 1944; Boivin, 1947).

The feasibility of separating nucleic acid derivatives by paper chromatography was demonstrated by Vischer & Chargaff (1948*a*) and by Hotchkiss (1948). A method for the quantitative analysis of ribonucleic acids (RNA) was developed by Vischer & Chargaff (1948*b*), and later applied to deoxypentose nucleic acids (DNA) (Chargaff, Vischer, Doniger, Green & Misani, 1949). This involves separation of the purines and the pyrimidines: the former are precipitated on methanolysis with dry hydrogen chloride, and liberation of the latter is completed by formic acid at 175°. The two groups are then estimated on separate chromatograms.

A simpler procedure for the analysis of RNA has been described by Smith & Markham (1950), who use a short hydrolysis in N-hydrochloric acid to liberate purines along with pyrimidine nucleotides, all of which are then separated on one-dimensional paper chromatograms. This method is not applicable to DNA, from which nucleotides or nucleosides cannot be obtained quantitatively by chemical hydrolysis because of the lability of the deoxy-sugar. In the method now described, DNA is hydrolysed by formic acid to purines and pyrimidines, which are separated on single paper strips. Preliminary results from this method have already been reported (Wyatt, 1950), and the identification and estimation of the pyrimidine 5-methylcytosine in DNA is described in the preceding paper (Wyatt, 1951).

Since this work was done, Daly, Allfrey & Mirsky (1950) have published analyses of DNA from a number of sources, based on separations obtained with starch columns. Their results differ from those now presented in that they failed to recognize 5-methylcytosine, and inclined to the view that the differences between various nucleic acids were not significant.

METHODS

Detection of the spots of chromatograms

For detection of the spots of purines and pyrimidines on paper chromatograms the photographic technique of Markham & Smith (1949) was used, which consists in making contact prints of the dried chromatograms on reflex document paper with filtered ultraviolet light of wavelengths 254 and 265 mμ.

Chromatographic solvents

Although purines and pyrimidines can be separated by various neutral, ammoniacal or weakly acid solvents (a number are listed by Markham & Smith, 1949), such solvents

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are not satisfactory for quantitative resolution of nucleic acid hydrolysates in amounts adequate for reliable estimation, because guanine 'tails' badly, owing to its low solubility. Many such solvents were tested. However, when Smith & Markham (1950) had shown that solvents containing relatively high concentrations of HCl could be used successfully on filter-paper chromatograms, systems of this type were tried. Increasing acidity has the effect of slowing the movement of all the substances and must be balanced by increasing the water content. The purines are held back more than the pyrimidines, and in solvents containing HCl stronger than about 1N, adenine moves more slowly than cytosine, reversing the positions they take in neutral systems. After many mixtures had been tested, that selected for resolution of the bases from DNA was an aqueous solution containing *iso*propanol 65% (v/v) and HCl 2·0N in the whole volume. This is made up as follows: to 65 ml. of absolute *iso*propanol (or 68 ml. of 95% *iso*propanol) at 16° add sufficient titrated conc. HCl to give 0·2 g.mol. of HCl, and water to 100 ml. The proportions are rather critical, and some empirical adjustment may be necessary; at higher temperatures the volume of *iso*propanol is reduced slightly. To obtain the best spacing of the spots the solvent should be used within a few days of mixing and the tank must be very well saturated with its vapour.

The R_F values of the common purines and pyrimidines in this solvent are given in Table 1, along with those of some nucleosides and nucleotides, to illustrate the effect on chromatographic movement of linkage to ribose and deoxyribose and of phosphorylation. These must be regarded merely as a guide to the relative positions of the spots, as absolute R_F's vary considerably.

Table 1. *R_F values in filter-paper chromatography of nucleic acid derivatives in* iso*propanol* (65%, *v/v*)-*water*-HCl (2·0N)

	R_F (Whatman no. 1 filter paper)
Purines:	
Guanine	0·25
Xanthine	0·25
Hypoxanthine	0·31
Adenine	0·36
Pyrimidines:	
Cytosine	0·47
5-Methylcytosine	0·55
Uracil	0·68
Thymine	0·77
Nucleosides and nucleotides:	
Cytosine riboside	0·50
Cytosine deoxyriboside	0·63
Cytidylic acid	0·61
Uridylic acid	0·79

Up to 75 μg. of each substance can be resolved from mixtures in 35 cm. movement of the solvent front. An incidental advantage of this solvent is that xanthine and hypoxanthine are estimated along with guanine and adenine respectively, so that the error caused by any deamination of the purines will be minimized. Before printing, the chromatograms must be dried until most of the HCl has evaporated. This can be done overnight by hanging them at room temperature, or for about 30 min. in a current of warm air, but excessive heat causes charring and must be avoided.

Estimation of the purines and pyrimidines

The procedure for estimation of the substances resolved on paper chromatograms is as follows. Triplicate spots of hydrolysate are applied to Whatman no. 1 filter paper from a micropipette (that used throughout these experiments contained 18·0 μl.); the chromatograms are run by the descending method until the front has moved about 35 cm. and are then dried and printed photographically. The positions of the spots are traced from the print on to the filter paper, and disks including them are cut out, along with blanks of equal size cut from a free 'lane' opposite the substances to be estimated; each is eluted in a test tube containing 5 ml. of 0·1N-HCl. After standing for several hours, the eluates are read at their absorption peaks against the corresponding blanks in the Beckman spectrophotometer.

When tested with solutions of pure substances, this method has given recoveries of 96–100%. The range of variation of replicate spots on one chromatogram and of subsequent chromatograms of one material is generally less than 5%; occasional greater discrepancies have been traced to remnants of ultraviolet-absorbing detergent or other dirt on the test tubes used for elution. A smaller error may result from the absorption of the silica Beckman cells themselves, which varies from cell to cell (cf. Morton, 1949). The set of cells used in this work have an absorption peak at 243 mμ. with an optical density averaging 0·014 when the three more opaque cells are read against the most transparent. Their absorption difference at 250 mμ. is sufficient to cause an error of up to 3% in estimations of guanine; this has been corrected for in results here presented.

A technique for the estimation of minor components, such as methylcytosine, by elution from one chromatogram and running again with another solvent, has already been described (Wyatt, 1951).

Table 2. *Ultraviolet absorption data on purines and pyrimidines in* 0·1N-*hydrochloric acid*

(mϵ is millimolecular extinction coefficient.)

Substance	Wavelength (mμ.)	mϵ	$E_{1\text{ cm.}}^{0\cdot001\%}$	$E_{1\text{ cm.}}^{0\cdot001\%}$ (Hotchkiss, 1948)
Adenine	260	13·0	0·96	1·01
Guanine	250	11·0	0·73	0·665
Uracil	260	7·9	0·705	0·72
Cytosine	275	10·5	0·95	0·91
Thymine	265	7·95	0·63	0·61
5-Methylcytosine	283	9·8	0·785	—

For use in estimation of the purines and pyrimidines separated by chromatography, extinction coefficients have been determined from pure samples. The sources of the material used for this are: guanine and uracil Hoffmann-La Roche; adenine, British Drug Houses Ltd.; thymine, commercial sample recrystallized; cytosine, isolated from herring-sperm nucleic acid, purified through the picrate and recrystallized; 5-methylcytosine, the gift of Dr A. Pircio, University of Chicago, purified through the picrate and

recrystallized. Each gave only one spot on chromatography except cytosine, in which a trace of methylcytosine persisted; this, however, was too small to cause significant error. The samples were dried, weighed and dissolved in 0·1 N-HCl for extinction measurement. Following Hotchkiss (1948), readings were taken at the nearest multiple of 5 mμ. to the wavelength of maximum absorption, except for methylcytosine. Results are given in Table 2, along with values calculated from the data of Hotchkiss for comparison; all except guanine agree within 5% with Hotchkiss's values.

Quantitative hydrolysis of deoxypentose nucleic acid

Hydrolysis with formic acid. The value of pure formic acid in the hydrolysis of nucleic acids was shown by Vischer & Chargaff (1948*b*), who found that it liberated pyrimidines with much less deamination than was caused by strong mineral acids. For RNA, however, 2 hr. at 175° were required for complete splitting of the pyrimidine ribosides, and in this time significant destruction of the purines had occurred. This led to their using separate hydrolyses for the purines and the pyrimidines.

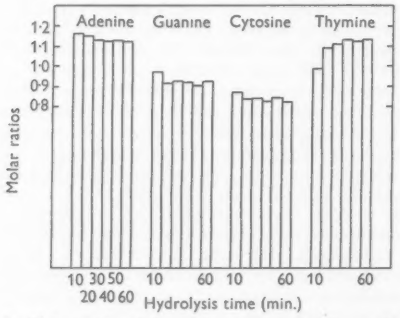

Fig. 1. Molar ratios of the purines and pyrimidines released from herring-sperm deoxypentose nucleic acid by hydrolysis in 98% formic acid at 175° for various lengths of time. For each hydrolysis time the yield of each base in molar units is expressed as a proportion of a total of 4·00.

It was known, however, that acid hydrolysis of DNA proceeds more rapidly than that of RNA, and in the hope of obtaining quantitative yields of all the bases from a single hydrolysis, shorter treatments with formic acid were tried. The purines are released from DNA by exceedingly short acid treatment, and of the pyrimidines cytosine is freed more quickly than thymine. At 165° in 98% formic acid recovery of cytosine reaches a maximum after 15 min., but the yield of thymine increases up to 2 hr. The results of a series of hydrolyses of herring-sperm DNA at 175° are shown in Fig. 1. Maximal yield of thymine is reached by 30 min., and there is no significant drop in recovery of any of the bases up to 60 min. (the higher initial ratios of adenine, guanine and cytosine are due to deficiency of thymine, since the four are calculated to constant totals). The experiment was repeated with a commercial thymus nucleic acid, with the same outcome. 30 min. at 175° in 98% formic acid therefore appears to give maximal yields of both purines and pyrimidines from DNA. As a further check on possible destruction of purines, the yield of adenine from DNA by this treatment was compared with that from 15 min. in aqueous N-HCl at 100°, and found to be the same.

To carry out this hydrolysis conveniently 1–30 mg. of DNA is weighed into a Pyrex tube of 13 mm. diameter, or a solution containing this amount is evaporated to dryness in the tube. Then 0·5 ml. of 98% formic acid is added and the tube is sealed off and heated in an electric muffle at 175° for 30 min. After cooling, the tube is opened cautiously in view of its contents being under pressure and the hydrolysate evaporated to dryness in the same tube. Aqueous N-HCl is then added in sufficient quantity to make a solution 3–4% (w/v) with respect to the original nucleic acid, allowance being made for the volume of the nucleic acid on the assumption that its specific volume is 0·6. The hydrolysate is allowed to dissolve and portions are applied to the chromatogram paper with a micropipette. It is important to use strong enough acid for dissolving the hydrolysate, as otherwise part of the guanine may remain out of solution.

Hydrolysis with perchloric acid. Since most of this work was done, Marshak & Vogel (1950) have reported quantitative hydrolysis of both RNA and DNA by 12 N-$HClO_4$ at 100° for 1 hr. This method has been tried. In the experiment summarized in Table 3, equal quantities of ox-spleen DNA were hydrolysed with formic acid as described above and with 72% (w/w) $HClO_4$ (11·1 N) at 100° for 1 hr., and analysed by chromatography simultaneously. The differences were small, and the total recovery of bases was nearly the same by each method. By repeating the comparative experiment, it was confirmed that $HClO_4$ gives consistently a slightly higher molar ratio of guanine and a lower one of thymine than formic acid; the reasons for this are not clear, but slight destruction of guanine by treatment with formic acid is probable.

Hydrolysis with $HClO_4$ has also been tried on RNA. A sample of the purified yeast nucleic acid which had been analysed by Smith & Markham (1950) was hydrolysed in

Table 3. *Recovery of bases from ox-spleen deoxypentose nucleic acid after hydrolysis with formic and with perchloric acids*

	Mol./atom P in hydrolysis with			Molar ratios* in hydrolysis with	
	HCOOH (*A*)	$HClO_4$ (*B*)	*A*/*B*	HCOOH	$HClO_4$
Adenine	0·257	0·259	0·99	1·13	1·12
Guanine	0·191	0·207	0·92	0·84	0·90
Cytosine	0·198	0·195	1·02	0·87	0·85
Thymine	0·254	0·250	1·02	1·12	1·08
Total	0·900	0·911	0·99	3·96	3·95

* Calculated to total 3·95, making 4·00 when 0·05 is added for 5-methylcytosine (cf. Table 8).

this way; the molar ratios found are compared in Table 4 with ratios calculated from Smith & Markham's (1950) published values by applying certain corrections recently determined by Dr R. Markham (personal communication). Considering that the two methods involve different hydrolyses, different chromatographic solvents, and the use of independently determined extinction values, the closeness of agreement is remarkable.

Table 4. *Composition of yeast ribonucleic acid hydrolysates*

	Molar ratios found by Smith & Markham (1950) (corrected—see Text)	Molar ratios found after hydrolysis with $HClO_4$ (means of 3 estimations)
Adenine	1·02±0·01*	1·04±0·01*
Guanine	1·19±0·01	1·22±0·005
Cytosine	0·83±0·02	0·82±0·01
Uracil	0·96±0·04	0·92±0·015

* Standard error of the mean.

Hydrolysis with $HClO_4$ is conveniently carried out as follows. To the air-dry nucleic acid in a small Pyrex tube is added a measured volume of 72% $HClO_4$ such that the concentration of nucleic acid in solution is 6–8%. The tube is closed with a glass stopper bound in place and is heated in a boiling-water bath for 1 hr. After cooling, the hydrolysate is diluted with an equal volume of water, and the tube is briefly centrifuged to sediment the residue of carbon. Samples can then be pipetted from the supernatant solution directly on to the chromatogram paper. In the *iso*propanol-aqueous HCl solvent, $HClO_4$ remains at the starting point and does not interfere with separation of the bases. Owing to the very hygroscopic nature of conc. $HClO_4$, it is advisable to determine the concentration of nucleic acid in the hydrolysate by estimation of P in samples taken at the same time as those taken for chromatography.

As a further check on possible destruction of the bases by either formic or perchloric acid, some experiments were performed to test the recovery of known amounts of pure bases added to nucleic acid and then subjected to hydrolysis. Table 5 shows the results of one such test. Portions of a solution containing adenine, guanine and thymine in 90% (w/w) formic acid (which is an excellent solvent for sparingly soluble bases such as guanine) were added to samples of ox-spleen DNA and evaporated to dryness, and the mixtures hydrolysed by both the formic acid and the $HClO_4$ techniques. Samples of the nucleic acid without the added bases were hydrolysed at the same time, and the quantities of the bases in the various hydrolysates and in the unhydrolysed solution of pure bases estimated by chromatography. P estimations on the hydrolysates gave a check on volume change by the $HClO_4$ solutions. The amounts recovered of the three added bases were all better than 95% by both methods of hydrolysis, so that little destruction during hydrolysis is indicated. It should be pointed out, however, that great accuracy cannot be expected from an experiment of this sort, which depends upon the comparison of three estimated quantities, each subject to experimental error, and the true yields may not be quite as nearly quantitative as these figures suggest. A previous similar experiment gave less satisfactory results, although recovery of each base was still better than 90%.

It is therefore concluded that essentially quantitative yields of both purines and pyrimidines from DNA can be obtained by treatment either with 98% formic acid at 175° for 30 min., or with 72% $HClO_4$ at 100° for 1 hr., and that the second method is suitable for RNA also. Since the latter method was not tried until its publication by Marshak & Vogel (1950), the former has been used in most of the analyses described in this paper. The use of $HClO_4$ has certain practical advantages, since the use of sealed tubes is not necessary, but difficulty has been experienced in hydrolysing impure nucleic acids with $HClO_4$, for the presence of protein interferes, retarding splitting of pyrimidine ribosides.

Preparation of nucleic acids

For standardization of the analytical technique two preparations of DNA were made by methods avoiding the use of acid, alkali or heat. One was prepared from ox spleen (calf thymus not being available) by a method similar to that of Mirsky & Pollister (1946). The minced fresh spleen tissue was washed three times with cold 0·15M-NaCl, then extracted with M-NaCl. Nucleoprotein was precipitated from the centrifuged extract by dilution with 6 vol. of water, then redissolved in M-NaCl and deproteinized by emulsification with $CHCl_3$-commercial octanol mixture (8:1, v/v) and centrifugation. After dialysis against water the nucleic acid was precipitated by addition of 1 vol. ethanol (90%, v/v) and washed successively with 90% (v/v) and

Table 5. *Recovery of added bases subjected to hydrolytic conditions in the presence of ox-spleen deoxypentose nucleic acid*

	μg./chromatogram spot			
Formic acid hydrolysis:	Adenine	Guanine	Cytosine	Thymine
Found in hydrolysed mixture	65·8	63·8	19·4	55·0
Accounted for by NA	30·7	25·4	19·3	28·4
Extra base found, by difference	35·1	38·4	—	26·6
Extra base added	36·6	39·2	0·0	26·3
Percentage recovery	96%	98%	—	101%
Perchloric acid hydrolysis:				
Found in hydrolysed mixture	67·2	67·2	19·4	53·1
Accounted for by NA	31·1	27·6	19·1	27·9
Extra base found, by difference	36·1	39·6	—	25·2
Extra base added	36·6	39·2	0·0	26·3
Percentage recovery	99%	101%	—	96%

absolute ethanol and ether. A sample for weighing was dried at 110° over P_2O_5 *in vacuo*.

DNA was prepared from herring testes by a similar procedure, except that papain was used for removal of the protamine, which fails to form a gel with $CHCl_3$. Some protein remained in this preparation.

The method of Mirsky & Pollister (1946) for preparation of crude nucleoprotein by extraction with M-NaCl and precipitation by dilution with water is applicable to most tissues (though not to mammalian sperm) and has been used in preparing most of the DNA preparations here studied. When the ratio of RNA to DNA in the original tissue is high, however, preparation of the latter in the pure state requires tedious reprecipitations accompanied by considerable loss. In making DNA from locusts, even when the minced tissue was washed four times with 0·15M-NaCl and the nucleoprotein twice precipitated with water, some RNA remained.

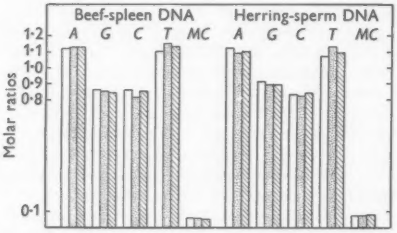

Fig. 2. Molar ratios of purines and pyrimidines from ox-spleen and herring-sperm deoxypentose nucleic acids, untreated (□), after treatment with N-NaOH (▦), and after treatment with ribonuclease (▧). *A*, adenine; *G*, guanine; *C*, cytosine; *T*, thymine; *MC*, 5-methylcytosine.

Since the fractionation of Schmidt & Thannhauser (1945) with N-NaOH has been widely used as an analytical procedure, it seemed worth while to discover whether this treatment might safely be applied to the preparation of DNA for analysis. Samples of the ox-spleen and herring-sperm DNA were dissolved in N-NaOH, left overnight at 37°, precipitated by acidification with glacial acetic acid and the addition of 1 vol. of ethanol, and analysed. In case this treatment might alter the composition by removal of traces of RNA, identical samples were incubated with ribonuclease and precipitated. The composition after each treatment is compared with the original composition in Fig. 2. The only significant change caused by the action of both alkali and ribonuclease on both nucleic acids is a slight increase in the ratio of thymine to the other bases, and this could easily result from the removal of a trace of contaminant RNA. The composition is otherwise not significantly altered, and the characteristic differences between the two nucleic acids are retained. Treatment with N-NaOH overnight at 37° has therefore been used wherever removal of RNA was necessary, and with preparations containing much RNA (such as nucleic acid from whole locusts) has been found much more effective than ribonuclease. Of the nucleic acids whose analysis is given below, NaOH was used in the preparation of those from bull sperm, ram sperm, rat-bone marrow, locusts and wheat germ.

The finding that the composition of DNA is unaltered by alkaline treatment does not, of course, diminish the importance of using mild methods of preparation when macromolecular properties are to be studied.

A sample of calf-thymus nucleic acid prepared by a modification of the method of Hammarsten (1924) was the gift of Dr K. Bailey, DNA from rat-bone marrow was prepared by Dr C. Lutwak-Mann, and that from ram sperm in collaboration with Dr T. Mann, to all of whom thanks are due.

Elementary micro-analyses

Total N was estimated by the micro-Kjeldahl method, and P by the colorimetric methods of Allen (1940) and Tisdall (1922).

RESULTS

In Table 6 are shown the results of an analysis of calf-thymus nucleic acid with respect to purines and pyrimidines (after hydrolysis with formic acid), P and N. The P and N accounted for by the estimated purines and pyrimidines have been integrated, the former on the assumption of simple polynucleotide structure, and the calculated values expressed as percentages of total P and N.

The results of similar analyses of other nucleic acids with respect to P and N are summarized in Table 7.

Table 6. *Composition of calf-thymus nucleic acid* (Na *salt*)

	% of dry wt.	μg. mol./mg. dry wt. Found	Calculated from bases	% of total accounted for	Molar* ratios
Adenine	10·00	0·741	—	—	1·11
Guanine	8·51	0·563	—	—	0·84
Cytosine	6·37	0·574	—	—	0·86
Thymine	9·46	0·751	—	—	1·13
Methylcytosine	0·46	0·037	—	—	0·056
Total bases	34·80	2·666	—	—	4·00
Phosphorus	9·4	3·03	2·67†	88	—
Nitrogen	14·5	10·36	9·86	95	—

* Calculated to an arbitrary total of 4·00.
† Assuming simple polynucleotide structure.

Table 7. *P and N contents of nucleic acid preparations with percentages of total P and N accounted for by estimated purines and pyrimidines*

Nucleic acid of	% of dry wt.		% of total accounted for by estimated bases (means of all experiments)	
	P	N	P	N
Calf thymus	9·4	14·5	90	94·5
Ox spleen	9·35	15·0	90	92·5
Ram sperm	7·4	15·9	88·5	68
Herring sperm	8·7	15·1	91	88
Wheat germ	9·7	14·9	84·5	92
Bull sperm	7·0*	—	90	—
Echinus sperm	8·6*	—	92	—

* Not specially dried.

The mean molar ratios of purines and pyrimidines in eight animal and one plant DNA are listed in Table 8, calculated in each case to an arbitrary total of 4·00. Where three or more independent analyses (separate hydrolyses and separate chromatograms) have been made, the standard error of the mean is shown.

Noting that Smith & Markham (1950) accounted for more than the total P of yeast RNA by their estimated bases, I have analysed a sample of their purified yeast nucleic acid by hydrolysis with perchloric acid, and although the ratios of the bases came very close to theirs (Table 4), the P accounted for by the bases was only 92 % of the total P. It seems most likely that both types of nucleic acid do contain bases and P in the 1:1 relationship usually assumed, and that the deficiency of estimated bases may be due to undiscovered error in the analytical techniques.

Significance of the molar ratios

The conclusion has been drawn by Chargaff, Zamenhof & Green (1950) that the composition of deoxyribonucleic acids varies in different species but not in different tissues of one species. The results of the present study tend to confirm this generalization and to strengthen it by the inclusion of 5-methylcytosine in the analyses.

Among the three bovine DNA preparations analysed those of thymus and spleen show no significant differences. The slightly increased purine/pyrimidine ratio in bull-sperm DNA as compared with the other two may be due to incipient autolysis, as the sperm was incubated with papain in an attempt to extract the nucleic acid before this was done with alkali; there is evidence that nucleases detach pyrimidine nucleotides preferentially (Overend & Webb, 1950). The methylcytosine ratio, however, is identical in the three bovine DNA preparations, whereas it is significantly different in each of the other nucleic acids analysed. As the ratios of the other bases are very similar in cow, sheep and rat DNA, the content of methylcytosine appears to be one of the most characteristic differences between nucleic acids from different species.

When more distantly related species are compared the divergences become greater. Herring DNA differs significantly from bovine DNA with respect to guanine as well as methylcytosine, and in locust and sea-urchin nucleic acids the differences are greater again.

Table 8. *Ratios of purines and pyrimidines from deoxypentose nucleic acids, after hydrolysis with formic acid*

Source of nucleic acid	No. of analyses	Molar ratios calculated to total 4·00				
		Adenine	Guanine	Cytosine	Thymine	Methylcytosine
Calf thymus	3	1·13±0·013	0·86±0·008	0·85±0·008	1·11±0·011	0·052±0·004
Ox spleen	7	1·13±0·006	0·85±0·006	0·84±0·011	1·13±0·008	0·054±0·002
Bull sperm	2	1·15	0·89	0·83	1·09	0·052
Ram sperm	3	1·15	0·88	0·84	1·09	0·039±0·001
Rat bone marrow	2	1·15	0·86	0·82	1·14	0·044
Herring sperm	6	1·11±0·015	0·89±0·009	0·83±0·006	1·10±0·021	0·075±0·002
Locusta migratoria (whole)	3	1·17±0·016	0·82±0·025	0·83±0·015	1·17±0·010	0·008±0·002
Echinus esculentus sperm	3	1·24±0·019	0·78±0·003	0·74±0·014	1·18±0·004	0·071
Wheat germ	2	1·06	0·94	0·69	1·08	0·23

DISCUSSION

The relation between bases and phosphorus

In all the nucleic acids analysed, more P is present than can be accounted for by assigning one phosphate radical to each molecule of estimated purine and pyrimidine. The fraction of the total P accounted for in this way averages 90 % (Table 7), and does not differ materially whether based on P estimated directly in samples of hydrolysate or on the P content of the original material. This discrepancy from 100 % seems greater than can be accounted for by destruction of the bases during hydrolysis, and suggests the presence of some phosphate additional to that in a simple polynucleotide structure. In an attempt to remove any loosely bound phosphate contaminant, a portion of ox-spleen DNA was reprecipitated ten times and analysed, but the fraction of total P accounted for by the bases was still only 91 %, so that little if any extraneous phosphate had been removed.

Remarkable, however, is the constant pattern of composition in all the animal DNA so far analysed (the 'molar ratios' of adenine and thymine being greater, and those of guanine and cytosine less than unity), in contrast with the widely divergent compositions of microbial DNA's (Vischer, Zamenhof & Chargaff, 1949; Smith & Wyatt, 1951). The only DNA of a higher plant so far analysed, that of wheat germ, has a pattern of composition similar to that of the animal nucleic acids, but is marked by an exceptionally high content of methylcytosine.

These characteristic ratios of the purines and pyrimidines in nucleic acids from different sources are not consistent with any simple theory of nucleic acid composition, but indicate a highly complex structure. In view of the localization of DNA in the chromosomes and the indications that DNA composition is more similar among related than unrelated species, it is tempting to speculate whether DNA composition may bear some direct relation to genetic structure.

There is very little evidence whether nucleic acids, as prepared, are homogeneous, or whether they contain mixtures of different molecular types whose total composition is constant from a given source. If the molecules in a given nucleic acid preparation are all of one type, the proportion of methylcytosine (e.g., 1 nucleotide in 75 in ox, 1 in 53 in herring, 1 in 500 in locusts) sets a minimum repeating unit of structure which, although less than the particle weight estimated for thymus nucleic acid by physical methods ($0{\cdot}5$–2×10^6, e.g., Cecil & Ogston, 1948), implies a remarkably elaborate molecule. Though this may be the case, it is equally possible that the preparation contains a number of particle types which, although differing in composition, are similar in size and net charge and so appear homogeneous when examined by electrophoresis or centrifugation.

SUMMARY

1. A micromethod has been devised for the estimation of the purine and pyrimidine bases in deoxypentose nucleic acids. Both purines and pyrimidines are released by a single hydrolysis with formic or perchloric acid and separated on a single paper chromatogram.

2. Extinction coefficients of the common purines and pyrimidines have been determined at their absorption maxima in the ultraviolet.

3. The composition of deoxypentose nucleic acid is unaltered by treatment with N-sodium hydroxide (Schmidt & Thannhauser, 1945) and this fractionation can satisfactorily be used for obtaining specimens free from ribonucleic acid for analysis.

4. Simple polynucleotides calculated from the estimated purines and pyrimidines account for about 90% of the total phosphorus and up to 94% of the total nitrogen of the various deoxypentose nucleic acid preparations analysed.

5. In analyses of deoxypentose nucleic acids from eight animal sources and from one plant the ratios of the purines and pyrimidines vary with the species source of the nucleic acid, but not with different tissues from one species. The content of 5-methylcytosine shows particularly characteristic variations.

I am indebted to the Anti-Locust Research Centre for supplies of locusts, and to Lord Rothschild for samples of bull semen and *Echinus* semen. I wish also to thank Dr R. Markham and Dr J. D. Smith for helpful discussion of the work. The work was carried out while I was on transfer of research from the Forest Insect Investigations Unit, Division of Entomology, Department of Agriculture, Canada, to the Agricultural Research Council Plant Virus Research Unit, Molteno Institute, Cambridge. This paper is Contribution No. 2702, Division of Entomology, Science Service, Department of Agriculture, Ottawa, Canada.

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DL-β-3-Oxindolylalanine (DL-Hydroxytryptophan)

1. SYNTHESIS

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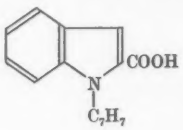
It is now generally assumed that the first series of reactions in the metabolism of tryptophan in mammals leads to kynurenine, the structure of which has been elucidated by Butenandt, Weidel, Weichert & Derjugin (1943). Kotake (1931, 1935), who, following its discovery by Matsuoka & Yoshimatsu (1925), carried out the first extensive investigations on kynurenine, suggested that β-oxindolylalanine (XVII) is an intermediate in the conversion of tryptophan to kynurenine and kynurenic acid; this hypothesis has been accepted by others (see Butenandt *et al.* 1943). β-Oxindolylalanine has not yet been found in mammalian tissues or excreta, but Wieland & Witkop (1940) obtained it from the hydrolysate of phalloidin, a toxic peptide from *Amanita phalloides*. Moreover, it was shown by Butenandt, Weidel & Becker (1940) that Wieland & Witkop's amino-acid was only slightly less active than kynurenine as precursor of the eye pigment of certain mutant strains of *Drosophila melanogaster*. It thus seemed likely that β-oxindolylalanine may also play a part in tryptophan metabolism in mammals, but no experiments to test this point have been carried out, largely owing to the fact that this amino-acid has so far been available in minute quantities only.

The occurrence of a tryptophan derivative containing a nuclear hydroxyl group in casein digests was reported by Abderhalden & Kempe (1907) and Abderhalden & Sickel (1924). Although these claims were later withdrawn (Abderhalden & Sickel, 1925), the earlier report stimulated Fischer & Smeykal (1923) to attempt the synthesis of oxindolylalanine. These authors condensed oxindole-3-aldehyde with hippuric acid, but obtained, instead of the expected azlactone, a substance which could not be converted to an amino-acid and which was suggested later by Horner (1941) to be an isatin derivative. Similar results were obtained by Gränacher & Mahal (1923) who attempted the condensation of oxindole-3-aldehyde with rhodanine. Julian, Pikl & Wantz (1935) condensed 1:3-dimethyloxindole with bromoacetal and converted the aldehyde obtained on hydrolysis by the Strecker method to β-(1:3-dimethyloxindolyl)alanine. This reaction, however, was unsuccessful with oxindole itself. Later Horner (1941) attempted the preparation of derivatives of oxindole suitably substituted in the 3-position; but no intermediate useful for the synthesis of the amino-acid was obtained. However, Witkop (1947) succeeded in oxidizing L-tryptophan with a mixture of acetic anhydride and hydrogen peroxide to a partially racemized oxindolylalanine. By comparison with the rotation of the material isolated from phalloidin, it was shown that the amino-acid obtained by Wieland & Witkop (1940) had the L-configuration. The method of Witkop (1947) gave apparently poor yields of pure product and does not lend itself, in our experience, to the preparation of large amounts of this amino-acid. We have therefore again attempted the synthesis of β-oxindolylalanine.

Various unsuccessful attempts at synthesis

It appeared that the mobility of the hydrogen atom in position 3 was the main reason why earlier attempts starting from 3-formyloxindole had failed. The latter reacts, as already emphasized by Gränacher & Mahal (1923), mainly in the enolic form. However, Julian *et al.* (1935) had reported that the sodium salt of 3-formyl-1-methyloxindole can be methylated to give a compound which has an *O*-methyl group in the 2-position and in which enolization is thus prevented. It was hoped to prepare the corresponding derivative of 1-benzyl-3-formyloxindole (V) which on reaction with hippuric acid might be expected to give an azlactone. Reduction of the intermediate *N*-benzyl compound might be expected to give the desired amino-acid. 1-Benzyloxindole (III) could not be prepared by the convenient Stollé synthesis, since treatment of *N*-benzylchloroacetanilide with aluminium chloride gives oxindole itself (see Stollé, 1930). III was therefore prepared by oxidation of 1-benzylindole-2-carboxylic acid (I) with hypobromite (Fischer & Hess, 1884; Colman, 1888; Michaelis, 1897). Good yields were obtained when this oxidation was carried out in a borate buffer pH 9·5–10·0. The bromine atoms in the resulting 3:3-*dibromo-oxindole* (II) show the same lability as in derivatives of bromo- or dibromo-malonate: they are readily reduced by iodide in acetic acid at room temperature. This method of debromination has been used for analytical purposes as well as for the preparative conversion of the dibromo-compound to 1-*benzyloxindole* (III). The

latter readily reacted with ethyl formate (Stollé, 1932) to give 1-benzyl-3-formyloxindole which from its reactions must be formulated as an enol (IV).



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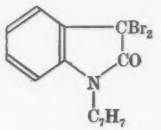

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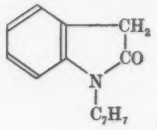

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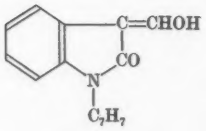

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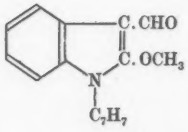
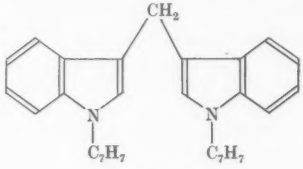


(V)

However, treatment of the sodium salt of (IV) with methyl iodide in acetone (Julian *et al.* 1935) produced an oil (possibly V) which could not be induced to crystallize. Attempts to condense the oil with hippuric acid under the usual conditions of the Erlenmeyer synthesis or with 2-phenyloxazolone in the presence of basic catalysts failed to give an azlactone.



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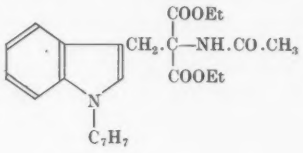

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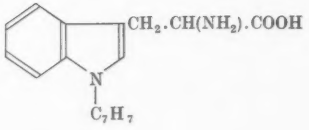

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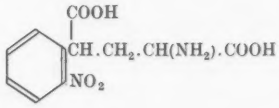


(IX)

We next attempted to oxidize a suitably substituted tryptophan derivative, since it appeared likely that the low yield of β-oxindolylalanine observed by Witkop was mainly due to the presence of a hydrogen atom in the 1-position of the indole nucleus. We therefore prepared DL-1-benzyltryptophan (VIII). The Mannich reaction with 1-benzylindole yielded mainly the methylene bis-compound (VI), whilst the reaction proceeded normally with 1-benzylindole-2-carboxylic acid. However, a modification of the method of Moe & Warner (1948) yielded (VIII) in a good yield. γ-Acetamido-γγ-dicarbethoxybutyraldehyde was condensed with benzylphenylhydrazine and the resulting hydrazone was cyclized. The ethyl α-acetamido-α-carbethoxy-β-(1-benzylindole-3) propionate obtained (VII) was hydrolysed first to α-*N*-acetyl-1-benzyltryptophan and then further to 1-benzyltryptophan. (In all names based on tryptophan, the atoms of the nucleus are numbered as in indole and those in the side chain lettered αβ as in alanine.)

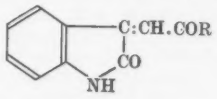
Oxidation of (VII) with hydrogen peroxide and acetic anhydride (peracetic acid) gave in small yield a crystalline product which apparently contained one more oxygen atom than would be required for an oxindole derivative. Its ultraviolet absorption spectrum was similar to those found with oxindole derivatives which had been exposed to autoxidation in alkaline solution (see Cornforth, Dalgliesh & Neuberger, 1951). The compound may be a dioxindole derivative. Oxidation experiments on α-*N*-

acetyl-1-benzyltryptophan using bromine were equally unsuccessful.

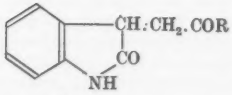
Having failed in these two approaches, we explored the possibility of synthesizing γ-(2-nitrophenyl)-glutamic acid (IX), reduction of which would have been followed by ring closure to oxindolylalanine. However, we were unable to obtain the desired nitro compound.

Synthesis of oxindolylalanine.

Lindwall & Maclennan (1932) showed that in the condensation of isatin with methyl ketones, 3-hydroxyoxindoles are formed first and these could easily be dehydrated to alkylideneoxindoles (X) which in turn may be reduced to alkyloxindoles (XI).

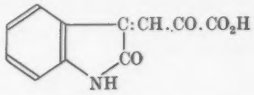


(X)

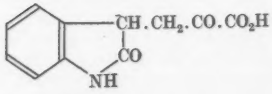


(XI)

We therefore attempted the condensation of isatin with ethyl pyruvate. When diethylamine was added to an equimolar mixture of these substances in ethanol, reaction was rapid and after acidification the deeply coloured 3-*isatylidenepyruvic acid* (XII) was isolated. The rapid hydrolysis of an ester grouping observed in this reaction is a little surprising, though α-keto acid esters are well known to hydrolyse in alkaline media very readily.

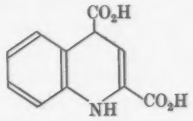


(XII)

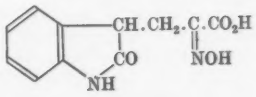


(XIII)

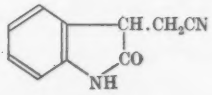
No indication of hydroxytryptophan formation was obtained when the acid (XII) was hydrogenated in the presence of ammonia, nor was any better result obtained when the addition of ammonia was withheld until one molecule of hydrogen had been absorbed. Attempts to isolate 3-oxindolylpyruvic acid (XIII) from the mixture obtained on catalytic hydrogenation of (XII) were defeated by the instability of the compound. It was, however, possible to prepare a *phenylhydrazone* of the α-keto acid. It is possible that the acid (XIII) rearranges easily to a dihydroquinoline (XIV) which polymerizes or undergoes dismutation. Reduction of the phenylhydrazones of acids (XII) and (XIII) either catalytically or with aluminium amalgam was tried, but was not promising.



(XIV)



(XV)

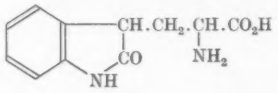


(XVI)

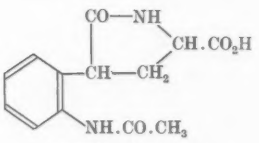
The next attempt was aimed at the oxime of (XIII). Reduction of isatylidenepyruvic acid (XII) with sodium dithionite to (XIII) occurred readily in aqueous solution; addition of hydroxylamine hydrochloride to the reaction mixture afforded the desired α-oximino-β-3-oxindolylpropionic acid (XV) in 55% yield on acidification. The success of this process is probably due to a masking of the keto group by the sodium bisulphite formed in the reduction, the pyruvic acid being thereby stabilized. On boiling with water the acid (XV) gave 3-oxindolylacetonitrile (XVI).

Reduction of the oximino group in the acid (XV) by aluminium amalgam did not proceed smoothly, but reduction with sodium amalgam in a slightly acid medium gave DL-oxindolylalanine (XVII) in 80–90% yield. The properties of the racemic amino-acid correspond closely with those given by Wieland & Witkop (1940) for the L-isomer. Optical resolution of the DL-compound presents difficulties which so far have not been overcome. The *N*-formyl, *N*-acetyl and *N*-benzoyl derivatives have been prepared by conventional methods. Among these only the acetyl derivative gave crystalline salts with brucine and (−)-α-phenylethylamine. Recrystallization of these salts gave no useful results. Fractions of different optical rotations were indeed obtained, but no controllable or progressive separation could be achieved.

When DL-hydroxytryptophan was boiled for a short time with dilute sodium or barium hydroxide and acetic anhydride was then added, a crystalline acid was isolated on acidification in a poor yield. This was found to be isomeric with the normal acetyl derivative and is probably the pyrrolidone (XVIII). The oxindole ring is opened on heating with alkali (Baeyer & Comstock, 1883) and β-oxindolylalanine behaves in an analogous manner (Wieland & Witkop, 1940). A crystalline brucine salt was obtained from this acid, but a preliminary attempt at optical resolution was unsuccessful.



(XVII)



(XVIII)

β-Oxindolylalanine can theoretically exist in two racemic forms, since, apart from the α-carbon atom of the side chain, C-3 of the oxindole nucleus is asymmetric. The optically active forms of dioxindole are rapidly racemized by a trace of alkali (McKenzie & Stewart, 1935), but no definite information is available on the mobility of the hydrogen atom in the 3-position of 3-alkyloxindoles. The behaviour of oxindolylalanine on crystallization can perhaps be attributed to the presence in solution of two diastereoisomers.

The ultraviolet absorption of oxindolylalanine and of related compounds is described in another paper (Cornforth *et al.* 1951). It should be mentioned here, however, that β-oxindolylalanine changes rapidly in alkaline solution and the value of the specific rotation given by Wieland & Witkop (1940) which was obtained in N-sodium hydroxide may have to be revised. The absorption data also suggest that the structure of the amino-acid is best represented by a lactam structure as in (XVII); the term hydroxytryptophan is therefore misleading and should be replaced by oxindolylalanine.

EXPERIMENTAL

All melting points are uncorrected.

Preparation of 1-*benzyl*-3-*formyloxindole*

NN-Benzylphenylhydrazine. Benzylation of phenylhydrazine in liquid NH_3 (Audrieth, Weisiger & Carter, 1941) or in benzene (Crowther, Mann & Purdie, 1943), with sodium and sodamide, respectively, yield besides the required material other products in small quantities. One of these was *NN'*-dibenzyl-*N*-phenylhydrazine hydrochloride, crystallized from ethanol, m.p. 195°; Franzen & Kraft (1911) give m.p. 186°. (Found: C, 74·0, H, 6·7; N, 8·6. Calc. for $C_{20}H_{20}N_2.HCl$; C, 74·0; H, 6·5; N, 8·6%.) The second by-product had m.p. 110° and was the benzylphenylhydrazone of benzaldehyde. (Found: C, 84·4; H, 6·7; N, 10·2. Calc. for $C_{20}H_{18}N_2$: C, 83·9, H, 6·3; N, 9·8%.) This compound has previously been produced by oxidation of dibenzylphenylhydrazine; in the present case autoxidation presumably occurred during the working up. The most convenient method, however, for the preparation of benzylphenylhydrazine was found to be that of Minunni (1892), which consists of heating carefully a mixture of 2 equiv. of phenylhydrazine with 1 equiv. of benzyl chloride. The benzylphenylhydrazine was distilled at 137–141°/0·8 mm., and then converted into the hydrochloride which is stable.

1-*Benzylindole-2-carboxylic acid.* This compound was prepared from the benzylphenylhydrazone of pyruvic acid as described by Antrick (1885). Cyclization was performed by heating the hydrazone (133 g.) which had been dissolved in ethanol (200 ml.) with 3N-HCl (1·5 l.) at 90° for 20 min. The solid formed on cooling was collected and dissolved in N-NaOH (1 equiv.). Insoluble material was filtered off and the alkaline solution was extracted with ether. On acidification the acid crystallized out; recrystallized from 50% (v/v) acetic acid, the m.p. was 194°. Yield 50% of theory.

3:3-*Dibromo*-1-*benzyloxindole.* A solution of 1-benzylindole-2-carboxylic acid (40 g.) in 0·45N-NaOH (250 ml.) was mixed with 0·2N-borate pH 9·6 (1 l.). To the cooled and mechanically stirred solution was added in ten equal portions over 30 min. a cooled solution (1 l.) of NaOBr (80 g. Br_2 dissolved in N-NaOH with cooling). The pH was kept between 9·5 and 10·0 by addition of N-acetic acid. After standing for 1 hr. at room temperature, the solid was collected, washed with water and dried. Yield was 80%. The material after successive recrystallization from acetic acid and ethanol had m.p. 158–159°. (Found: C, 47·3; H, 3·1; N, 3·8. $C_{15}H_{11}ONBr_2$ requires C, 47·3; H, 2·9; N, 3·7%.) The two Br atoms are readily replaced by H; 38·1 mg. were dissolved in acetic acid and KI (100 mg.) was added. After standing at 37° for 1·5 hr. the liberated I_2 was titrated with 0·1N-$Na_2S_2O_3$. 4·1 ml. were consumed; $C_{15}H_{11}ONBr_2$ requires 4·0 ml.

1-*Benzyloxindole.* To a solution of the dibromo compound (40 g.) in boiling ethanol (750 ml.) was added Zn dust (40 g.) (treated successively with 5% (w/v) $HgCl_2$, water and ethanol) and conc. HCl (100 ml.). Reaction started at once and the solution boiled. After 0·5 hr. more Zn (30 g.) and conc. HCl (30 ml.) were added and the solution boiled under reflux for 1·5 hr. The solution was concentrated under reduced pressure to dryness and the residue dissolved by addition of ether and water. The ethereal layer was separated and the aqueous layer was repeatedly extracted with ether. The combined ethereal solutions were dried and the solvent removed. The remaining oil was distilled at 150–155°/0·3 mm. The distillate soon crystallized; the m.p. after recrystallization from a small quantity of light petroleum (b.p. 100–120°) was 65°. Yield was 75%. On repeated crystallization from this solvent or from aqueous ethanol the m.p. was raised to 67°. (Found: C, 80·3; H, 5·8; N, 6·5. $C_{15}H_{13}ON$ requires C, 80·7; H, 5·7; N, 6·3%.) 1-*Benzyloxindole* was also prepared and in slightly better yield by reduction of the dibromo-indole with KI in acetic acid.

1-*Benzyloxindole-3-aldehyde.* *N*-Benzyloxindole (13·74 g.) and ethyl formate (5·55 g.) dissolved in warm ethanol (60 ml.) were added to a hot solution of 2·1N-NaOEt in ethanol (36 ml.). The mixture, which solidified at once, was allowed to stand for 10 min. and then cooled to 0°. The Na salt was decomposed by addition with shaking of N-HCl (100 ml.) and water (300 ml.). The solid was filtered and dried. Yield was 90%. Successive recrystallization from $CHCl_3$-light petroleum (b.p. 30–40°) and benzene gave material of m.p. 175°. (Found: C, 76·1; H, 5·4; N, 5·6. $C_{16}H_{13}O_2N$ requires C, 76·5; H, 5·2; N, 5·9%.) The aldehyde gives a blue colour with ethanolic $FeCl_3$; it is soluble in ethanol and $CHCl_3$ and moderately soluble in benzene. The *phenylhydrazone* was prepared by boiling a solution of equimolar amounts of the aldehyde and phenylhydrazine acetate in ethanol and had m.p. 186–187°. (Found: C, 77·4; H, 5·3; N, 12·5. $C_{22}H_{19}ON_3$ requires C, 77·4; H, 5·6; N, 12·3%.)

Attempted preparation of 1-*benzyl-2-methoxyindole-3-aldehyde.* To a solution of 1-benzyloxindole-3-aldehyde (11·166 g.) in a mixture of ethanol (150 ml.) and dry ether (75 ml.) was added 1·75N-sodium ethoxide (26·5 ml.). After cooling the mixture to −10° the precipitate which was formed was filtered off and washed with cold ethanol and dry ether. The Na salt was dried by heating for 1 hr. at 100°/0·1 mm. It was then suspended in dry acetone (250 ml.) containing methyl iodide (30 g.) and the mixture boiled under reflux for 8 hr. The clear solution, which did not give a $FeCl_3$ reaction, was concentrated to low bulk; no solid was obtained. Water was added and the oil extracted with ether. On drying the ethereal solution and removal of the solvent, an oil was obtained which could not be induced to crystallize. Heating of the oil with hippuric acid (1 equiv.), acetic anhydride (4 equiv.), and anhydrous sodium acetate (0·5 equiv.) did not apparently yield an azlactone. Treatment of the oil with 2-phenyloxazolone in ethanol in the presence of β-picoline or pyridine was equally unsuccessful.

The Mannich reaction with 1-*benzylindole derivatives*

Mannich reaction with 1-*benzylindole.* 1-Benzylindole (Antrick, 1885) (2 g.) was submitted to the Mannich reaction using the procedure of Snyder & Eliel (1948). After completion of the reaction, the mixture was made alkaline, extracted with ether and the ether solution extracted with dilute acid. The aqueous extract on addition of NaOH and extraction with ether eventually gave an oil in poor yield which solidified on standing. Recrystallization from light petroleum (b.p. 40–60°) gave 1-*benzyl-3-dimethylaminomethylindole*, m.p. 52–54°. (Found: N, 10·4. $C_{18}H_{20}N_2$ requires N, 10·6%.) The base gave a *picrate*, dark-yellow prisms from ethanol, m.p. 122°. (Found: N, 14·1. $C_{18}H_{20}N_2.C_6H_3O_7N_3$ requires N, 14·2%.) The original ether extract on evaporation gave as the main product of the reaction a pinkish solid. Recrystallization from a large volume of methanol gave colourless crystals, m.p. 137°, identified as 3:3′-*methylene-bis*(1-*benzylindole*). (Found: C, 87·0; H, 6·2; N, 6·9. $C_{31}H_{26}N_2$ requires C, 87·3; H, 6·1; N, 6·6%.)

Mannich reaction with 1-*benzylindole-2-carboxylic acid.* To a cooled solution of 3·2 ml. dimethylamine (33% aqueous solution) and glacial acetic acid (5 ml.) was added 0·9 ml. 40% formaldehyde solution. After chilling, the indole derivative (2·5 g.) was added and the mixture shaken. As no apparent reaction took place, a further 2 ml. acetic acid were added and the mixture heated on a boiling-water bath for 1·5 hr., diluted with water and extracted with ether. The aqueous solution deposited crystals of the Mannich base which were recrystallized from methanol; m.p. 211° (decomp.). (Found: N, 8·9. $C_{19}H_{20}O_2N_2$ requires 9·2%.) The compound owing to its zwitterionic structure is fairly soluble in water and does not form an insoluble picrate.

1-*Benzyltryptophan*

Benzylphenylhydrazone of ethyl α-*acetamido-δ-aldehydo-*α-*carbethoxybutyrate.* To ethanol (65 ml.) containing a small amount of Na was added ethyl acetamidomalonate (15·2 g.). To the cooled solution acrolein (4·5 g.) was added slowly with shaking. The acrolein had been prepared immediately before the experiment by hydrolysis of acrolein diacetate with the calculated amount of water containing a trace of mineral acid. The mixture was set aside in the dark for 1 week, and then treated with benzylphenylhydrazine (13·9 g.) and glacial acetic acid (2 ml.), heated to boiling, diluted with water (15 ml.), filtered and left in the dark for 18 hr. The *hydrazone* separated in the form of white needles, m.p. 127–128° (22·5 g.; 62% yield based on acrolein). On recrystallization from ethanol m.p. was 131°. (Found: C, 66·3; H, 6·6; N, 9·3. $C_{28}H_{31}O_5N_3$ requires C, 66·2; H, 6·8; N, 9·3%.) The product became coloured if exposed to light.

Cyclization of the hydrazone. A mixture of the crude hydrazone (18·5 g.), water (120 ml.) and conc. H_2SO_4 (5·6 ml.) was refluxed with vigorous stirring for 3 hr. On standing a reddish gummy precipitate separated. The mixture was diluted with water, extracted with ether, and the ethereal solution, after re-extraction with water, was dried and concentrated. The residue was taken up in ethanol and the solution filtered. From this solution separated 14·7 g. crystalline material, m.p. 120–122°. Recrystallization from ethanol gave *ethyl* α-*acetamido-*α-*carbethoxy-*β(1-*benzylindole-3*)*propionate* of m.p. 123°. (Found: C, 68·9; H, 6·9; N, 6·2. $C_{25}H_{28}O_5N_2$ requires C, 68·8; H, 6·6; N, 6·4%.)

α-N-*Acetyl*-1-*benzyltryptophan.* The ester (4·9 g.) was refluxed with 2·5N-NaOH (22 ml.) for 3 hr. The solution was cooled and acidified with 2N-HCl (35 ml.). After standing at 0° the *malonic acid* (4·65 g.) was filtered off and dried. Melting point was 127° (decomp.). (Found: N, 6·9. $C_{21}H_{20}O_5N_2$ requires N, 6·7%.) The acid was decarboxylated by refluxing for 2 hr. with water (30 ml.) containing enough ethanol to give a clear solution. The solution was filtered and cooled. The *acetamido acid* first separated as an oil which later crystallized in the form of needles. Yield was 2·9 g. The material had, after recrystallization from 50% ethanol, m.p. 156°. (Found: C, 70·8; H, 5·8. $C_{20}H_{20}O_3N_2$ requires C, 71·4; H, 5·9%.)

1-*Benzyltryptophan.* The acetyl derivative (2·0 g.) was refluxed with 2N-NaOH (10 ml.) for 24 hr. The solution was acidified with acetic acid. On standing at 0° the amino-acid crystallized; yield was 1·4 g. It was recrystallized from aqueous ethanol and separated as *monohydrate*, m.p. 214° (decomp.). (Found: C, 69·7; H, 6·3. $C_{18}H_{18}O_2N_2.H_2O$ requires C, 69·3; H, 6·4%.) Drying to constant weight at 100–180° at 60 mm. over $Mg(ClO_4)_2$ gives the anhydrous amino-acid. (Found: C, 73·1; H, 6·2; N, 9·7. $C_{18}H_{18}O_2N_2$ requires C, 73·5; H, 6·1; N, 9·5%.)

Oxidation experiments with H_2O_2. Most of these experiments were carried out with (1-benzylindolyl)-acetamidomalonate. If more than 1 equiv. H_2O_2 was used, only brown

gummy or amorphous products were obtained. When 1 equiv. H_2O_2 was employed, a crystalline substance was obtained which had a composition which indicated that two atoms of O had entered the molecule, much of the starting material being recovered.

To an ice-cold solution of the acetamidomalonate (6·5 g.) in acetic anhydride (25 ml.) was added 1·52 ml. 9·85M-H_2O_2 (1 equiv.). After 10 min. the flask was removed from the ice bath and the temperature allowed to rise to 20°. The flask was then cooled again and allowed to warm to 20°, the cycle being repeated several times. After standing at 0° for 18 hr. the mixture was poured on to ice and after decomposition of the anhydride the product was filtered off, washed with water, taken up in hot ethanol and allowed to cool to 0°. A small amount of crystalline material was isolated, m.p. 200°. The filtrate on dilution with water gave successive crops of material with m.p. 105–110°. These were crystallized and identified as starting material. The material which separated first was recrystallized twice from ethanol to give needles of m.p. 201°. Analysis showed that two atoms of O had been taken up. (Found: C, 64·7; H, 6·2; N, 6·0. The original material with addition of one O atom, $C_{25}H_{28}O_6N_2$ requires C, 66·4; H, 6·2; N, 6·2%; with addition of two O atoms, $C_{25}H_{28}O_7N_2$ requires C, 64·1; H, 6·0; N, 6·0%.) The absorption spectrum showed a maximum at 248·5 mμ. and a subsidiary peak at 304 mμ. The absorption curve corresponded closely with that of oxindole which had been allowed to oxidize in alkaline solution and was then acidified; this showed a maximum at 242·5 mμ. and a subsidiary peak at 303 mμ.

Oxidation experiments with hypobromite. These were carried out in borate buffer as described above for benzylindolecarboxylic acid. Only brown amorphous products were obtained, from which no crystalline compounds could be isolated either directly or after treatment with NaI in acetic acid.

Synthesis of β-oxindolylalanine

3-*Isatylidenepyruvic acid.* A mixture of isatin (29·4 g.), ethyl pyruvate (22 ml.) and ethanol (60 ml.) was stirred rapidly and cooled in an ice bath. When the temperature had fallen to 10°, diethylamine (20 ml.) was added during 9 min.; the temperature rose to 15–18°. After a further 3 min. unreacted isatin was removed by rapid filtration, and washed with a little 25% (v/v) ethanol. To the filtrate 2N-HCl (180 ml.; previously chilled) was added immediately. Crystallization was induced by scratching and was encouraged to proceed as rapidly as possible by stirring and shaking. If the solution became turbid a little more ethanol was added. After about 1 hr. the crystals were collected, washed with 25% (v/v) ethanol, and air-dried. The yield was 16–20 g. It was found unprofitable to work up the mother liquors.

The product is a dihydrate. It is unstable in solution and recrystallization is wasteful; hence we used it directly for further work. A sample crystallized from aqueous dioxan in lustrous plum-coloured prisms. 3-*Isatylidenepyruvic acid* is easily soluble in ethanol, acetone and dioxan; sparingly in water and ether. The Na salt separates readily from aqueous solution, especially when excess alkali is present. The acid gives pale-yellow solutions in aqueous $NaHSO_3$ or H_2SO_3. It decomposes at about 210° without melting. (Found for air-dried material: C, 53·2; H, 4·7; loss of weight after drying at 80°/15 mm., 14·2, 14·5. $C_{11}H_7O_4N.2H_2O$ requires C, 52·2; H, 4·3; $2H_2O$, 14·2%. Found after drying at 80°: C, 60·8; H, 3·2. $C_{11}H_7O_4N$ requires C, 60·8; H, 3·2%.) The *phenylhydrazone*, prepared in aqueous acetic acid, crystallized from much ethanol in starlike aggregates of red needles, m.p. 191·5–193° (decomp.). (Found: C, 66·2; H, 4·4; N, 13·9. $C_{17}H_{13}O_3N_3$ requires C, 66·4; H, 4·2; N, 13·7%.)

3-*Oxindolylpyruvic acid phenylhydrazone.* This product was obtained in poor yield by two methods: (*a*) isatylidenepyruvic acid was shaken in aqueous suspension with PtO_2 and H_2 until somewhat more than 1 mol. of H_2 had been absorbed; the almost colourless solution was treated with phenylhydrazine acetate; (*b*) isatylidenepyruvic acid was reduced with $Na_2S_2O_4$ as described below, and the solution treated with phenylhydrazine. The *phenylhydrazone* crystallized from ethanol in pale-yellow needles, m.p.190–191° (decomp.). (Found: C, 66·1; H, 5·6. $C_{17}H_{15}O_3N_3$ requires C, 66·0; H, 4·9%.)

α-*Oximino-β-(3-oxindolyl)propionic acid.* 3-Isatylidenepyruvic acid (23·2 g.) was stirred with water and cooled in ice during the addition (5 min.) of $Na_2S_2O_4$ (15·9 g.). Stirring was continued until the dark-coloured acid had disappeared. The solution was then boiled for 4 min., frothing being countered by use of a roomy flask and addition when necessary of a little ether. The hot solution was treated with charcoal and filtered; hydroxylamine hydrochloride (12 g.) was dissolved in the cooled filtrate. 2N-NaOH was now added to neutralize the solution and then to keep it neutral as oximation proceeded; after about 1 hr. the solution was acidified to congo red and kept at 0°. The crystalline precipitate was collected next day and dried *in vacuo*. The yield was 13·8 g. It was recrystallized (with about 75% recovery) by dissolving in boiling methanol (10 ml./g.), treating with charcoal, filtering and adding an equal volume of water. The *acid* (XV) separated in small, colourless needles, m.p. 172–173° (decomp.; variable with rate of heating) after drying *in vacuo*. It tended to crystallize as a trihydrate. (Found: for an air-dried specimen: loss at 80°, 18·7, 18·8. $C_{11}H_{10}O_4N_2.3H_2O$ requires $3H_2O$, 18·7%). This became anhydrous in a vacuum desiccator. (Found for vacuum-dried material: C, 56·2; H, 4·8; $C_{11}H_{10}O_4N_2$ requires C, 56·2; H, 4·3%.)

3-*Oxindolylacetonitrile.* The oximino acid (XV) (1·75 g.) was refluxed for 1·5 hr. in 25 ml. water. After cooling, the crystals (1·15 g.) were collected; the mother liquor was only faintly acid. Recrystallization from water and then ethanol gave the *nitrile*, colourless rhombic prisms m.p. 162–165°. (Found: C, 69·3; H, 4·7; N, 16·1. $C_{10}H_8ON_2$ requires C, 69·8; H, 4·6; N, 16·3%.)

DL-α-*Amino-β-(3-oxindolyl)propionic acid.* (DL-*Hydroxytryptophan.*) The recrystallized anhydrous oximino-acid (XV) (5·1 g.) was stirred with 100 ml. water and cooled in ice during the gradual addition (about 0·5 hr.) of 2·3% sodium amalgam (118 g.; freshly prepared). Conc. HCl was added *pari passu* to keep the reaction mixture slightly acid. When the amalgam had reacted, a few drops of the liquor were treated with 2:4-dinitrophenylhydrazine in 2N-HCl: if any precipitate appeared on warming, more amalgam and acid were added. The filtered and neutralized aqueous solution was evaporated rapidly at low pressure to small bulk. Crystallization was usually spontaneous and was more rapid if the solution was kept hot for a time. After keeping overnight at 0° the crystals were collected (4·2–4·8 g.). This product had m.p. 241–242° (decomp.). Recrystallization was best effected by taking up quickly in

a relatively large volume of O_2-free hot water, filtering (if necessary, after addition of charcoal), and concentrating to small volume at low pressure with a N_2 leak. The *amino-acid* (XVII) separated very slowly in small plates forming a hard white crust; m.p. 244–245° (decomp.). (Found: C, 59·6, 59·9; H, 5·7, 5·8; N, 13·0, 12·7, 12·9. $C_{11}H_{12}O_3N_2$ requires C, 60·0; H, 5·5, N, 12·7%.) The colours with ninhydrin and Millon's reagent, and the precipitation with mercuric and silver ions, were as described by Wieland & Witkop (1940) for the L-amino-acid.

β-Oxindolyl-α-formamidopropionic acid. DL-Oxindolylalanine (2 g.) was dissolved in 15 ml. anhydrous formic acid (treated with P_2O_5) and acetic anhydride (5 ml.) was gradually added with stirring at 58°. The solution was allowed to stand at 18–20° for 0·5 hr. and then poured into water (150 ml.). The aqueous solution was extracted with three portions of ethyl acetate (total 500 ml.). The ethyl acetate solution was dried and concentrated to dryness. The resulting oil slowly crystallized in the form of plates. Yield was 1·15 g. The material was recrystallized from water (5 ml.) and had m.p. 172°. (Found: C, 58·2; H, 5·3; N, 13·2. $C_{12}H_{12}O_4N_2$ requires C, 58·1; H, 4·8; N, 13·2%.)

The acetyl derivative was obtained by dissolving the amino-acid (0·5 g.) in N-NaOH (2·4 ml.) and adding acetic anhydride (1·5 ml.). When all anhydride had reacted the mixture was evaporated at low pressure. The residue was taken up in water and again concentrated; 0·24 ml. of conc. HCl was added and evaporation continued to dryness in a vacuum over KOH. The crystalline residue was washed with water; yield was 0·55 g., m.p. 208–210°. Recrystallization from water gave DL-*α-acetamido-β-(3-oxindolyl)propionic acid*, in colourless prisms, m.p. 209–210°. (Found: C, 59·6; H, 5·9; N, 10·7; equiv. 264. $C_{13}H_{14}O_4N_2$ requires C, 59·5; H, 5·4; N, 10·7%; mol.wt. 262.) The acid gave a yellow-brown colour with Millon's reagent.

DL-*α-Benzamido-β-(3-oxindolyl)propionic acid.* This was obtained by shaking the amino-acid (0·5 g.) with 1·3 N-$NaHCO_3$ (1·8 ml.) and benzoyl chloride (0·9 ml.). The solution was acidified and the precipitate extracted, after drying, with light petroleum to remove benzoic acid. Recrystallization from acetone and aqueous acetone gave colourless clustered rods, m.p. 153–154° after drying at 110°. (Found: N, 8·3. $C_{18}H_{16}O_4N_2$ requires N, 8·6%.)

3-(2′-Acetamidophenyl)-2-pyrrolidone-5-carboxylic acid (XVIII). DL-Oxindolylalanine (110 mg.) was refluxed with N-NaOH (1·5 ml.) for 6 min. The cooled solution was treated with acetic anhydride (0·4 ml.). Isolation of the product was carried out as described for the 'normal' acetyl derivative (above). The new *acid* (25 mg.; m.p. 227–228°) crystallized from water in rosettes of needles, m.p. 230°. (Found: C, 59·8; H, 5·7; equiv. 261. $C_{13}H_{14}O_4N_2$ requires C, 59·5; H, 5·4%; mol.wt. 262.) It gave no colour with Millon's reagent.

SUMMARY

Various possible routes for the synthesis of β-oxindolylalanine have been explored. A satisfactory synthesis in three stages from isatin has been worked out.

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β-3-Oxindolylalanine (Hydroxytryptophan)

2. SPECTROSCOPIC AND CHROMATOGRAPHIC PROPERTIES

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The synthesis of β-oxindolylalanine from isatin and ethyl pyruvate is described in the preceding paper (Cornforth, Cornforth, Dalgliesh & Neuberger, 1951). During the course of this work the chromatographic behaviour of an apparently pure specimen of the amino-acid on filter paper was examined; it was found in the earlier preparations that the synthetic substance gave, apart from the main spot, a weak subsidiary spot indicating the presence of a small amount of material with hydrophilic characteristics. This finding led to a more extensive investigation covering other compounds metabolically related to tryptophan or oxindolylalanine.

The second part of this paper is concerned with the ultraviolet absorption of oxindolylalanine and various other substituted oxindoles and the effects of changes of pH and oxidation on the absorption spectra. A comparison of the behaviour of 3-methyloxindole and that of the new amino-acid confirmed the structure assigned to the latter on the basis of the synthesis. Finally, the structure of phalloidin is discussed on the basis of its reported absorption spectrum as compared with that of oxindolylalanine.

Chromatography

EXPERIMENTAL

General. Paper chromatography was carried out in the usual way using Whatman no. 4 paper. The compounds were applied as 0·2% solutions with the exception of kynurenic acid which was an approximately saturated solution containing considerably less than 0·2%.

Colour reactions used on paper. With ninhydrin, oxindolylalanine and kynurenine gave purple colours, whilst with tryptophan a greyish-blue colour was produced. Kynurenine, but none of the other three compounds, gave a red colour on diazotization with nitrous fumes and subsequent coupling with alkaline β-naphthol. On spraying with 2% (w/v) *p*-dimethylaminobenzaldehyde in 5% (w/v) HCl (Ehrlich's reagent) kynurenine gave an orange colour immediately, oxindolylalanine a pale-yellow colour and tryptophan a violet colour that developed much more slowly. Millon's reagent gave with tryptophan a pale-yellow colour immediately on spraying, whilst with oxindolylalanine an orange spot developed more slowly. On drying at about 70° the latter became deep orange and the tryptophan spot became pale orange.

It was shown by Wieland, H. & Witkop (1940) that oxindolylalanine responds to the colour reaction of Folin & Denis (1912) for phenols; this test has now been applied to paper chromatograms. The phosphomolybdic-tungstic acid solution was sprayed on the previously dried chromatogram, the paper was allowed to dry and then sprayed with 2N-Na_2CO_3. Oxindolylalanine gave a blue colour and tryptophan a pale-green colour, the colours sometimes appearing before the carbonate spraying. It was found important to use an all-glass sprayer when using the Folin-Denis reagent. With kynurenine no colour was produced.

Fluorescence on illumination with ultraviolet light. Kynurenic acid did not give a colour with any of the reagents tried, but it was clearly visible when illuminated with ultraviolet light. The light source used was an 'Osira' lamp (General Electric Company Ltd.) fitted with a Wood's glass envelope; the lamp emitted mainly between 3000 and 3500 A. Kynurenic acid on paper gave a blue-green fluorescence and kynurenine a strong pale-blue fluorescence. Tryptophan and oxindolylalanine did not fluoresce when acidic solvents were used for irrigation. However, when alkaline solvent mixtures had been used, spots fluorescing weakly on ultraviolet illumination appeared with both amino-acids. These spots did not coincide with the spots revealed on subsequent spraying with ninhydrin and are considered to be due to decomposition products formed during the prolonged exposure to alkaline solvents.

RESULTS

R_F values of oxindolylalanine, tryptophan, kynurenine and kynurenic acid

Many solvent systems were examined and the results obtained with some of these are shown in Table 1. Owing to the instability under alkaline conditions of most of the compounds investigated, basic solvent systems are not to be recommended; moreover, they offer no advantage with respect to resolution. In acidic systems kynurenic acid appeared to be faster than tryptophan, oxindolylalanine and kynurenine, which had similar R_F values with all the usual solvent mixtures examined. Tryptophan, kynurenine and kynurenic acid could readily be separated, especially when some of the more specific colour tests described above were applied; but none of the usual solvent systems was found capable of resolving all the four substances. Anomalous retardation on development of the filter-paper chromatograms with water (Synge & Tiselius, 1949) was shown by all three amino-acids, but the R_F values were again very similar.

Table 1. *R_F values of tryptophan, oxindolylalanine, kynurenine and kynurenic acid found with different solvent systems*

(In all but one experiment, the ascending technique of Williams & Kirby (1948) was used. The descending technique of Consden, Gordon & Martin (1944) was employed in the experiment using phenol-NH_3. Where figures are given in parentheses, the solvent was allowed to run off the paper and the figures represent the distances (in cm.) travelled.)

Solvent mixture		R_F value			
Composition	Reference	Tryptophan	β-Oxindolyl-alanine	Kynurenine	Kynurenic acid
n-Butanol-acetic acid-water (4:1:5)	Partridge (1946)	0·54	0·52	0·50	0·66
Phenol containing 0·3% NH_3 (w/v) and a trace of NaCN	Consden *et al.* (1944)	(19·2)	(17·9)	(16·9)	—
Collidine-2:4-lutidine-water (1:1:2)	Dent (1948)	0·79	0·84	0·86	0·00
n-Butanol-benzyl alcohol saturated with water (1:1)	—	0·225	0·235	0·22	—
n-Butanol-propionic acid-water (50:25:35)	Calvin & Benson (1949)	0·56	0·53	0·50	—
n-Butanol-propionic acid-water (4:1:5)	—	0·45	0·41	0·36	—
n-Butanol-formic acid-water (4:1:5)	—	0·65	0·60	0·57	—
Amyl alcohol-pyridine-water (35:35:30)	de Verdier & Ågren (1948)	0·48	0·42	0·38	0·41
Water	Synge & Tiselius (1949)	0·68	0·75	0·78	0·74

Resolution and differentiation of tryptophan, oxindolylalanine and kynurenine was readily obtained when formaldehyde was incorporated into an acidic solvent mixture. Thus, with a homogeneous mixture formed from *n*-butanol, glacial acetic acid, 40% (w/v) formaldehyde solution and water (5:1:1:3 by vol.), kynurenine on subsequent treatment with ninhydrin gave a brown spot with R_F 0·77, oxindolylalanine gave a purple spot with R_F 0·63 and tryptophan gave two yellow spots with R_F values 0·68 and 0·93 respectively. In a mixture these spots were separate and could be readily distinguished. The slow-moving tryptophan spot corresponded to that given by 2:3:4:5-tetrahydro-β-carboline-4-carboxylic acid under the same conditions.

Chromatographic investigation of the purity of synthetic oxindolylalanine

Early preparations of oxindolylalanine which had been recrystallized and gave correct elementary analyses, gave on paper chromatography with various solvent mixtures two spots. The major spot had R_F values (reported in Table 1) close to those of tryptophan, whilst the minor spot corresponded to a more hydrophilic substance with R_F values which ranged with the acidic solvent mixtures between 0·09 and 0·28. In later preparations the second slower-moving spot was not observed.

This minor constituent of the early preparations reacted on paper with ninhydrin but not with the reagents of Millon, Ehrlich or Folin & Denis, and to obtain further information about its nature an attempt was made to isolate it on a column of paper pulp using the procedure of Campbell, Work & Mellanby (1951). From 1·5 g. of crude oxindolylalanine there was thus obtained 15 mg. of material, which did not melt below 350°, but charred. Analysis of this material (found: C, 53·0; H, 6·7; N, 5·8%) was not inconsistent with its being a tetrahydroquinoline derivative, but in view of the small amount available its exact nature was not established. In any case, the amount obtained from the chromatographic separation shows that it did not constitute more than 1–2% of even the earliest preparation.

Absorption Spectra

EXPERIMENTAL

All melting points are uncorrected.

Preparation of compounds. The preparation of β-oxindolylalanine and of 1-benzyloxindole is described by Cornforth *et al.* (1951). Oxindole was purified by repeated crystallization from water followed by sublimation *in vacuo*; it had m.p. 126°. 3-Methyloxindole was prepared by cyclization of β-propionylphenylhydrazine (Brunner, 1896, 1897); after steam distillation, recrystallization from water and sublimation *in vacuo*, it had m.p. 124°. 3:3-Dimethyloxindole was prepared from β-*iso*butyrylphenylhydrazine (Brunner, 1897) and purified in a similar manner; it had m.p. 152°. 1:3:3-Trimethyloxindole was prepared from α-methyl-β-*iso*butyrylphenylhydrazine (Brunner, 1897).

The product after distillation at 140–145°/16 mm. was converted into the $HgCl_2$ complex (Julian, Pikl & Boggess, 1934) which was crystallized successively from ether and aqueous ethanol; it had m.p. 125°; Julian *et al.* give 122·5° for the m.p. of the $HgCl_2$ complex. On decomposition with 2N-NaOH, extraction with ether and removal of the solvent, 1:3:3-trimethyloxindole was obtained as a colourless oil, b.p. 98–99°/1 mm.

Dioxindole was prepared by reduction of isatin with $Na_2S_2O_4$ (Marschalk, 1912) and was purified by repeated crystallization from benzene; it had m.p. 166–168°.

Measurements of absorption spectra. Most of the compounds were dissolved in water to give 10^{-3}–10^{-4}M-solutions. 1:3:3-Trimethyloxindole and 1-benzyloxindole were first dissolved in methanol; water or NaOH was then added to give a final concentration of 20% (v/v) methanol. The absorption spectra were measured as soon as possible after making up the solutions. Any solution not under observation was kept stoppered and in the dark. No special precautions were taken to exclude air. In the experiment in which the rate of change of molecular extinction coefficient of 3-methyloxindole at 340 mμ. with time was measured, the material was dissolved in water and an equal volume of 0·2N-NaOH added at zero time. A similar technique was employed with other compounds in measuring the immediate changes of absorption on addition of alkali.

The measurements were made with the Beckman spectrophotometer Model DU, employing 1 cm. cells and a hydrogen lamp.

RESULTS

Absorption spectra in neutral and acid solution

The absorption spectra of oxindole, 1-methyloxindole and 1:3:3-trimethyloxindole have been measured by Ramart-Lucas & Biquard (1935) who found that the spectra of these three compounds were similar, all having a maximum ($\log \epsilon = 4{\cdot}0$) at 250 mμ. All oxindole derivatives examined by us had maxima in neutral or acid solutions in positions which varied between 248 and 252 mμ. (Table 2); the ε values ranged from 7250 to 9000. At short wavelengths there was a minimum at 225 mμ. with ε ranging between 3000 and 3500. At longer wavelengths absorption fell sharply (Figs. 1–3), but all oxindole derivatives examined had a 'shoulder' between 270 and 285 mμ. with ε values falling from 2250 to 1000. This 'shoulder' was somewhat less marked in acid solution, but otherwise the spectra were almost identical at pH 1 and 7. The spectra of oxindolylalanine, its α-*N*-acetyl derivative (Fig. 6) and 3-methyloxindole were very similar in neutral solution (Figs. 1 and 6).

Changes of absorption spectra in alkaline solution

Immediate changes. All oxindole derivatives possessing an imino-hydrogen atom such as oxindole, 3-methyloxindole and 3:3-dimethyloxindole showed on addition of alkali an immediate shift of the absorption maximum amounting to about 8 mμ. This instantaneous change was followed in the case of oxindole and of 3-methyloxindole by a much more marked, but progressive alteration of the spectrum. The immediate shift of the maximum on addition of alkali did not occur with the two derivatives not possessing an imino-hydrogen atom, namely 1:3:3-trimethyloxindole and 1-benzyloxindole. However, with the latter, there were observed progressive changes similar to those found with other oxindole derivatives, containing one or two hydrogen atoms in the 3-position. The immediate shift which appeared on addition of alkali was seen most clearly with 3:3-dimethyloxindole (Fig. 2), where it was not obscured by secondary changes. Fig. 3 shows that with 1:3:3-trimethyloxindole the spectra in acid and alkaline solution were identical.

Progressive changes. When oxindole was left in 0·1N-sodium hydroxide for several hours, there was found intense absorption at wavelengths below 245 mμ., a maximum at 256·5 mμ. (ε = 7950) and a second peak at about 365 mμ. (ε = 2400). This spectrum did not change any further and resembled that of isatin. Similar changes were also found with 1-benzyloxindole.

Addition of alkali to a solution of 3-methyloxindole produced a change in the spectrum extending over about 2 hr., the appearance of a new peak at 340–344 mμ. being most marked. The rate of change, as measured by the increase of extinction at 340 mμ., is shown in Fig. 4. The spectra of oxindolylalanine

Table 2. *Positions of maxima and minima (mμ.) and the corresponding extinction coefficients of various oxindole derivatives*

	Solvent							
	Water				0·1N-HCl			
Substance	$\epsilon_{max.}$	$\lambda_{max.}$	$\epsilon_{min.}$	$\lambda_{min.}$	$\epsilon_{max.}$	$\lambda_{max.}$	$\epsilon_{min.}$	$\lambda_{min.}$
Oxindole	7680	248·5	3450	225·0	8600	248·5	3000	223·5
3-Methyloxindole	—	—	—	—	8000	249·0	3400	226·0
3:3-Dimethyloxindole	7680	248·5	3400	225·0	8090	248·5	3000	223·5
1:3:3-Trimethyloxindole	8950	252·5	—	—	—	—	—	—
1-Benzyloxindole	8470	251·0	3400	227·5	—	—	—	—
β-Oxindolylalanine	7250	250·0	3250	227·5	8450	251·0	3570	227·5
α-*N*-Acetyl-β-oxindolylalanine	—	—	—	—	7300	252·0	3000	224·5
Dioxindole	—	—	—	—	(*a*) 5300 (*b*) 1450	(*a*) 252·0 (*b*) 292·0	(*a*) 3800 (*b*) 1100	(*a*) 231·5 (*b*) 276·0

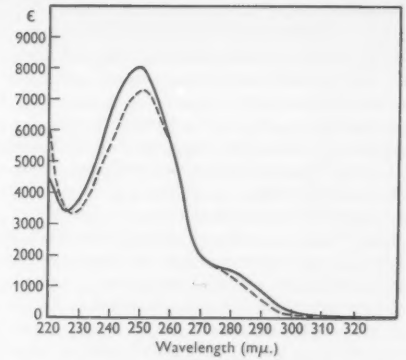


Fig. 1. Absorption spectra of β-oxindolylalanine, – – –, and of 3-methyloxindole, ——, in water.

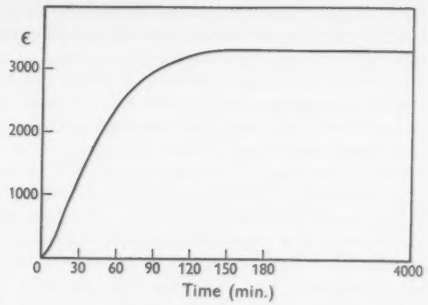


Fig. 4. Rate of increase with time of molecular extinction coefficient at 340 mμ. of 3-methyloxindole in 0·1 N-NaOH.

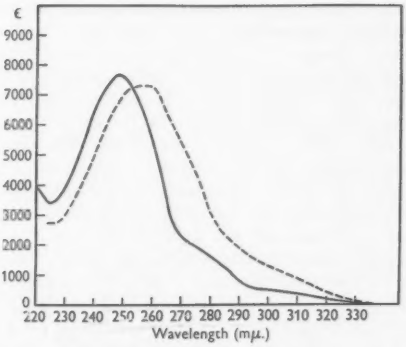


Fig. 2. Absorption spectra of 3:3-dimethyloxindole in water, ——, and in 0·1 N-NaOH, – – –.

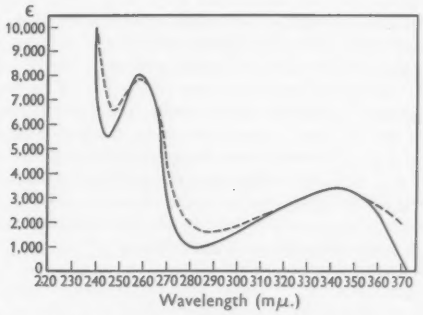


Fig. 5. Absorption spectra of β-oxindolylalanine, – – –, and 3-methyloxindole, ——, in 0·1 N-NaOH. Spectra were measured on solutions which had been left standing at room temperature for 4 hr.

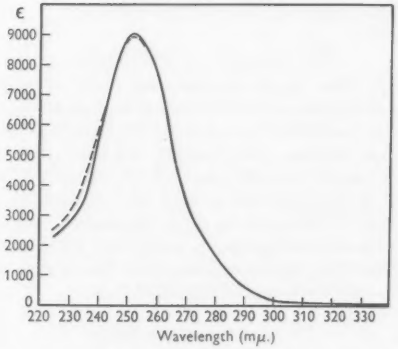


Fig. 3. Absorption spectra of 1:3:3-trimethyloxindole in neutral 20% (v/v) methanol, ——, and in 0·1 N-NaOH containing 20% (v/v) methanol, – – –.

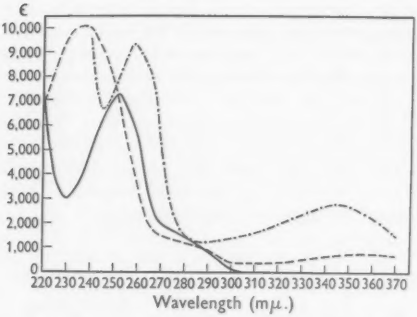


Fig. 6. Absorption spectra of α-*N*-acetyloxindolylalanine in 0·1 N-HCl, ——, in 0·1 N-NaOH, –·–·–), and in 0·1 N-HCl after exposure to 0·1 N-NaOH for 4 hr., – – –.

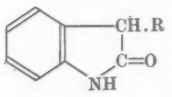
and its acetyl derivative, after exposure to 0·1N-sodium hydroxide for 4 hr., were almost identical with that of 3-methyloxindole measured under similar conditions (Figs. 5 and 6). All three spectra showed two maxima, one at 340–344 mμ. and the other at about 258 mμ., and in addition intense absorption at wavelengths below 245 mμ.

When a solution of 3-methyloxindole in 0·1N-sodium hydroxide was left to stand for 4–16 hr. and acidified until the concentration of hydrochloric acid was 0·1N, the spectrum was indistinguishable, from that of a solution (Fig. 1) that had never been made alkaline at all. But exposure to alkali for longer periods produced irreversible changes, as shown by the shift of the maximum (in acid) from 250 to 240 mμ. and a marked increase of absorption at wavelengths below 240 mμ. With oxindolylalanine and its acetyl derivative the changes brought about by exposure to alkali could not be reversed by acidification. This is shown in Fig. 6 for the acetyl derivative. With the free amino-acid the changes, as observed on the acidified solution, were even more marked. A solution of oxindolylalanine which had been left in 0·1N-sodium hydroxide for 18 hr. and was then acidified to pH 1·0 showed marked absorption in the near ultraviolet with a maximum at 360 mμ. ($\epsilon = 1600$) and intense absorption below 260 mμ. without an apparent maximum. This irreversible change may be due to oxidation to a dioxindole derivative; but dioxindole itself gives a somewhat different spectrum (Table 2).

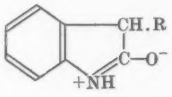
DISCUSSION

The structure of oxindolylalanine

The resemblance between the absorption spectrum of the synthetic amino-acid and those of other oxindole derivatives and particularly the close similarity between the spectrum of oxindolylalanine exposed to alkali for some time and that of similarly treated 3-methyloxindole, leave no possible doubt that the structure assigned to the new compound is correct. As already pointed out by Ramart-Lucas & Biquard (1935) the fact that the spectrum of oxindole is very similar to that of 1-alkyl-3:3-dialkyl- or 1:3:3-trialkyl-oxindoles indicates that oxindoles generally are best represented by formula (I) and the resonance structure (II); the enol form or the lactim form do not appear to be important contributors to the structure of oxindole.



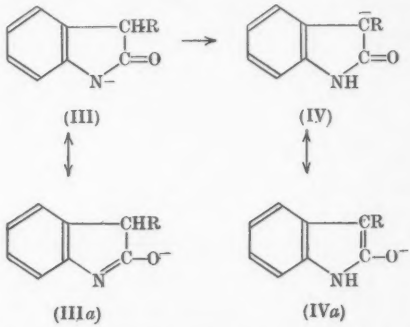
(I)



(II)

Changes on addition of alkali

The primary and instantaneous changes in spectral absorption on addition of alkali appear to depend on the availability of a hydrogen atom in the 1-position and not on the presence of a hydrogen atom in the 3-position. It would thus appear that ionization of oxindole as an acid consists primarily of a removal of the imino-hydrogen as a proton giving (III) and its resonance hybrid (III*a*). On the other hand, the secondary and progressive changes in absorption consisting of the appearance of new bands at 340 or 260 mμ. are associated with the presence of at least one hydrogen atom in the 3-position. Oxindole itself is readily oxidized in alkaline solution to dioxindole and isatin, and the spectral changes observed in this case are almost certainly associated with such an oxidation. With 3-methyloxindole the slow spectral changes on addition of alkali are at least at first reversible. These are best explained by a prototropic change (III → IV), similar to that found in ordinary enolization.



The enolate (IV) or (IV*a*) appears to be readily oxidized and this probably explains the irreversible changes found with the 3-alkyloxindoles.

The purity of oxindolylalanine

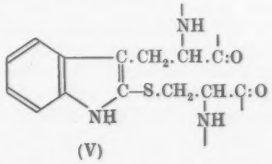
Both the spectrophotometric and chromatographic experiments have shown that oxindolylalanine is unstable in solution, particularly in an alkaline medium. The presence of a secondary spot in the paper chromatograms of the earlier preparations of the new amino-acid may be due to the presence of an impurity in the crystalline material, but the chromatographic, analytical and spectrophotometric results suggest that the amounts of such a contaminant is very small.

The structure of phalloidin

The data on the ultraviolet absorption of oxindolylalanine reported here have a bearing on the constitution of phalloidin. This toxic substance from

Amanita phalloides is a cyclic polypeptide and yields on hydrolysis alanine, cysteine, *allo*hydroxyproline and oxindolylalanine (Wieland & Witkop, 1940). Of the four amino-acids isolated, only oxindolylalanine absorbs light to any extent above 250 mμ. Two absorption curves of this polypeptide have been published; the first by Wieland, H. & Hallermayer (1941), who report two maxima in close proximity at 257 and 251 mμ. with log ϵ values of 4·31 and 4·35 respectively, a minimum at about 275 mμ. and a further maximum at 290 mμ. (log $\epsilon = 4{\cdot}0$). Wieland, T. (1949) reports a maximum at 290 mμ. (log $\epsilon = 4{\cdot}1$), a minimum at 257 (log $\epsilon = 3{\cdot}5$) and intense absorption at shorter wavelengths. The discrepancy of the two curves in the region below 275 mμ. is not easy to explain, but both papers report a maximum at 290 mμ. with a high extinction coefficient. The similarity of the spectra of oxindolylalanine and its acetyl derivative suggests that incorporation of this amino-acid into a peptide will not affect its absorption spectrum greatly. The finding, therefore, that phalloidin has a maximum at 290 mμ. instead of 250 mμ. must mean that either this peptide contains another light-absorbing structure not yet isolated, or that the oxindole ring is modified in phalloidin. Wieland, H. & Witkop (1940) believed that they had accounted for all the constituents of phalloidin and it is unlikely that a compound, which would have to be present in relatively large amounts to account for the high extinction coefficient, has been overlooked. Thus, it is probable that the oxindole ring is not present in phalloidin, but arises by hydrolysis of the peptide.

Wieland, H. & Witkop (1940) also reported that, provided oxygen is excluded during the hydrolysis of phalloidin, a positive nitroprusside reaction is obtained, indicating the presence of cysteine. On the other hand, phalloidin itself does not yield a precipitate of lead sulphide on treatment with alkaline plumbite (Lynen & Wieland, U., 1937). It thus seems that the thiol group like the oxindole group is masked in the peptide itself. It may therefore be suggested that phalloidin contains the structure (V) which might be expected to give an absorption spectrum of the same type as indole.



(V)

This type of structure on hydrolysis with acid might yield oxindolylalanine and cysteine.

SUMMARY

1. The R_F values of β-oxindolylalanine, tryptophan and kynurenine were measured employing various solvent mixtures. With the usual systems resolution was poor; however, separation was obtained with a butanol-acetic acid mixture containing formaldehyde.

2. The ultraviolet absorption spectra of a number of oxindole derivatives including β-oxindolylalanine have been measured. Addition of alkali was found to produce various reversible and irreversible changes which were correlated with the mode of substitution.

3. A comparison of the reported absorption spectra of phalloidin with those of oxindole derivatives indicates that the oxindole structure is not present as such in phalloidin.

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On the Role Played by Formate in Serine Biosynthesis

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Evidence for the conversion of glycine to the α- and carboxyl carbons of serine in rat-liver homogenates has been obtained by Winnick, Moring-Claesson & Greenberg (1948) and evidence for the existence of the same reaction in live rats has been given by Sakami. Sakami also found that formate, which had been administered to the rats along with glycine, served as a source for the β-carbon of serine; the possibility, that this incorporation was brought about via carbon dioxide, was excluded.

Sakami suggested that the process was the reverse of that postulated by Shemin (1946) for the conversion of serine to glycine

$$(HCOOH + CH_2NH_2COOH \rightarrow CH_2OHCHNH_2COOH).$$

The simultaneous incorporation of the two substances does not necessarily imply, however, that the incorporations are due to a condensation between glycine and formate or even between some derivatives of these. The carbons from the two substances might be incorporated by entirely independent processes. Sakami (1949*a*, *b*) reported the conversion, *in vivo*, of labile methyl groups and of the α-carbon of glycine to the β-carbon of serine. This latter conversion was also observed *in vitro* by Siekevitz & Greenberg (1949); they also obtained results with rat-liver slices which made them suggest that the transformation of the α-carbon of glycine into the β-carbon of serine proceeded via formate. These findings, together with the demonstration of an incorporation of formate-carbon into the 2- and 8-positions of uric acid (Sonne, Buchanan & Delluva, 1948), raise the question of whether formate is normally produced in intermediary metabolism. In particular, it might be asked whether the α-carbon of glycine and the carbon of labile methyl groups enter the β-position of serine via formate and whether formate should be considered the only immediate precursor of the β-carbon of serine. Further, it might be considered a possibility that the oxidation of formate in the animal body was brought about through incorporation into serine.

As an approach to these problems, the *in vitro* incorporation of formate carbon into serine has been studied. Slices and cell-free homogenates of rat liver, known to form serine from glycine, were used. The incorporation of formate carbon into the free serine of these preparations was measured following incubation with ^{14}C-labelled formate. In parallel experiments formation of serine from glycine was checked by incubation with carboxyl-^{14}C-labelled glycine. It was found that glycine and formate are both incorporated into serine by liver slices, but in homogenates glycine but not formate is incorporated; it appears, therefore, that formate is not the only or is not the immediate precursor of the β-carbon of serine. Related results were very recently reported by Siekevitz & Greenberg (1950). Ethionine was found to inhibit the formation *in vitro*, presumably in rat-liver slices, of formate from the α-carbon of glycine, while leaving the incorporation of glycine into serine unaffected.

Since it was further found that formate is oxidized at an appreciable rate in rat-liver homogenates, the conclusion can also be drawn that an incorporation of formate into serine is not a necessary step in its oxidation in rat liver. The oxidation of formate in preparations of rat-liver homogenates has been further studied in an attempt to characterize the enzyme systems responsible for this process.

EXPERIMENTAL

Isotope preparations

Sodium formate containing ^{14}C (giving 70,000 counts/min./μmol. under the prevailing counting conditions) was synthesized by the procedure of Melville, Rachele & Keller (1947). The formic acid content as determined by the microcolorimetric method of Grant (1948) accounted for 97% of the steam-distillable acids.

Carboxyl-^{14}C-labelled glycine (58,000 counts/min./μmol.) was prepared from acetate (Olsen, Hemingway & Nier, 1943). After four recrystallizations the product had a constant melting point (254–255° compared with an authentic sample which had m.p. 255–256°); a paper chromatogram contained practically all the activity within the glycine spot.

Tissue preparation and incubation

Homogenates of rat liver were prepared in the cold from 1 part of liver and 2·5 parts of medium and freed from unbroken cells by low-speed centrifugation (about 200 *g* for 5 min.). The media employed were: (1) the standard medium (*SM*) of Winnick *et al.* (1948) and (2) a bicarbonate-free medium (*BFM*) of the following percentage composition: KCl, 0·65; NaCl, 0·25; $MgSO_4.7H_2O$, 0·06; K_2HPO_4, 0·18; glucose, 0·10; sodium citrate, 0·03. The pH was adjusted to 7·3 and 100 ml. mixed with 2 ml. 1% $CaCl_2$ just before use. Rat-liver slices were cut free-hand in the cold.

Incubations were carried out by shaking in flasks at 38° in an atmosphere of 5% CO_2–95% O_2, when *SM* was used, and in 100% O_2, when *BFM* was employed. Usually 2–3 ml. homogenate were used, and there was no dilution other than by a small volume of the solution of radioactive compound. In the case of slices an amount representing 65–85 mg. dry wt. was suspended in 5 ml. of *SM*. In both cases nearly equimolar amounts of labelled formate or glycine, namely 2·3 μmol. $H^{14}COONa$ (~160,000 counts/min.) or 2·93 μmol. $CH_2NH_2{}^{14}COONa$ (~171,000 counts/min.), each contained in 0·2 ml. solution, were added to the flasks just before the incubation; 2·93 μmol. non-radioactive glycine were added together with the $H^{14}COONa$ and 2·3 μmol. non-radioactive formate together with the $CH_2NH_2{}^{14}COONa$. In some of the later experiments formate and glycine, both labelled with ^{14}C, were added to the same flask, as earlier experience had shown the activity in serine to be located exclusively in the β-carbon after incubation with $H^{14}COONa$ and in the carboxyl group after incubation with $CH_2NH_2{}^{14}COONa$. In most cases 1 mg. DL-serine was added to each flask before the start of the incubation to trap the serine synthesized.

Isolation of free serine

At the end of the incubation DL-serine (250 mg.) was added as a carrier to the homogenates and to suspensions of liver slices. The proteins were removed from the whole homogenates and from the tissue slice medium (plus wash water of slices) by heat coagulation at pH 5 and filtration. The filtrates were treated with norite and subsequently evaporated to about 2·5 ml. Ethanol (2 vol) was added to produce crystallization in the cold. After six or seven similar recrystallizations and repeated removal by centrifugation of substances insoluble in hot water, a product was obtained which had m.p. 209–211° almost identical with that of authentic DL-serine (210–211°), and contained practically all the ^{14}C activity in the serine-glycine spot of a paper chromatogram.

Separate experiments, in which 3 μmol. $CH_2NH_2{}^{14}COONa$ and 2 mmol. DL-serine were dissolved in water or in deproteinized solution from homogenates, showed that about 2% of the glycine activity was not removed from serine by the crystallization procedure mentioned above. However, this incomplete separation does not invalidate the results since less than 0·05% of the activity appeared in the CO_2 (representing the carboxyl group of serine) when this serine was degraded by periodate oxidation.

Degradation of serine and counting

The periodate method devised by Sakami (1948) was employed by which each of the three carbons of serine are obtained separately as CO_2. The CO_2 was precipitated as $BaCO_3$ (10–20 mg.) and plated on filter-paper disks. All counts were corrected to zero layer thickness. At least 2000 counts were recorded. Counts/min. lower than 2 above background were considered insignificant; in such cases the incorporation is stated as zero.

Determination of formate oxidation in homogenates

To determine the extent of the formate conversion to CO_2 in homogenates, CO_2 formed in some of the above experiments with formate was absorbed in KOH in the centre cup of the Warburg vessels used for incubation. However, for the closer study of this process in homogenates and preparations of these, another technique was adopted, mainly to allow constant cyanide concentrations to be maintained during incubation. Filtering flasks (50 ml. capacity) were used. The side tube was provided with a rubber cap and the mouth with a two-hole rubber stopper with inlet and outlet glass tubes which were connected by rubber tubes to a compressed gas source and a CO_2 absorber respectively.

The homogenate preparation was incubated in the flasks which were continuously shaken in a Warburg bath. When the effect of cyanide was tested the rubber tubes were kept clamped during incubation; otherwise a steady stream of the appropriate gas was directed through the flasks and absorbers during incubation. The rubber caps served for injections of $H^{14}COONa$ at the beginning of the incubation period and of H_2SO_4 at the end. To measure the total ^{14}C activity of the CO_2 absorbed, the KOH solution of the CO_2 absorber was subsequently diluted with a suitable Na_2CO_3 solution and portions of the mixture used for $BaCO_3$ plating. Bicarbonate-free medium was used in these experiments.

RESULTS

The percentages of the total activity, added as $CH_2NH_2{}^{14}COOH$ or $H^{14}COOH$, which could be recovered in the free serine at the end of the incubation periods are shown in Tables 1 and 2. All results entered under a particular experimental number were obtained with the same liver tissue.

As can be seen from Table 1 a considerable conversion of glycine as well as formate to serine takes place in liver slices, even though formate is incorporated to a lesser extent than glycine.

In the case of homogenates the situation is different (Table 2). There the incorporation of glycine/mg. tissue used in the preparation is of the same order of magnitude as in slices, but no incorporation at all could be demonstrated for formate, although formate of high activity was used.

In some of the above experiments with $H^{14}COONa$ in homogenates a considerable fraction of the activity was recovered in the respiratory carbon dioxide (cf. Table 2, Exps. 7 and 8).

Some of the results of separate experiments in which the formate-carbon dioxide conversion was studied under varying conditions appear in Tables 3, 4 and 5. They indicate that: (1) The enzyme system involved could not be sedimented by 2×30 min. centrifugation at about 20,000 *g* (Table 5); it was further found that a suspension of washed mitochondria showed little activity. (2) The enzyme activity is abolished by anaerobic conditions (Table 3). (3) The enzymic activity could be reduced by ageing and dialysis, and restored to its previous level by addition of coenzyme I (Co I) (Table 5). (4) The enzymic activity was inhibited by cyanide (Table 4); it was further found that this inhibition could not be overcome by methylene blue in molar concentrations up to seven times that of cyanide.

Table 1. *Incorporation of* $CH_2NH_2{}^{14}COOH$ *and* $H^{14}COOH$ *into serine in rat-liver slices*

(In all cases the slices were suspended in 5 ml. *SM*.)

Exp. no.	Incubation time (min.)	Labelled substances: Glycine* (μmol.)	Labelled substances: Formate* (μmol.)	DL-Serine added before incubation (μmol.)	Percentage of added activity found in serine: Carboxyl carbon	Percentage of added activity found in serine: β-Carbon
2	75	2·93	—	0	6·3	—
	75	—	2·3	0	—	1·16
3	90	2·93	—	10	9·6	—
	90	—	2·3	10	—	1·19
4* *a*	90	2·93	2·3	10	11·0	1·57
b	90	2·93	2·3	10	10·3	1·73

* In Exp. 4*b* 200 μg. aminopterin was added before the incubation, while no such addition was made in 4*a*. As seen from the figures this does not influence the glycine or formate incorporation.

Table 2. *Incorporation of* $CH_2NH_2{}^{14}COOH$ *and* $H^{14}COOH$ *into serine in cell-free homogenates of rat liver*

Exp. no.	Medium used	Homogenate used (ml.)	Incubation time (min.)	Labelled substances: Glycine (μmol.)	Labelled substances: Formate (μmol.)	DL-Serine added before incubation (μmol.)	Percentage of added activity found in serine: Carboxyl carbon	Percentage of added activity found in serine: β-Carbon	Percentage of added activity found in respiratory CO_2
1	*SM*	1·8	75	2·93	—	0	3·33	—	—
		1·8	75	—	2·3	0	—	0	—
6	*SM*	3·0	90	2·93	—	10	7·0	—	—
		3·0	90	—	2·3	10	—	0·1	—
7	*BFM*	2·5	120	2·93	—	10	7·8	—	—
		2·5	120	—	2·3	10	—	0	87
8	*BFM*	2·5	120	—	4·6	0	—	0	88
		2·5	120	—	4·6	150	—	0	85
		2·5	120	2·93	—	150	7·6	—	—

SM = Standard medium. *BFM* = Bicarbonate-free medium.

Table 3. *Influence of aerobic and anaerobic conditions on the conversion of formate to* CO_2 *in rat-liver homogenate*

(3 ml. samples of a final supernatant, obtained by three successive centrifugations (10 min. each at about 5000 *g*) of a homogenate (1 part of liver + 2 parts of medium), were incubated at 38° for 60 min. with 0·2 ml. solution containing 7 μmol. $H^{14}COONa$ with 16,000 counts/min.)

	Incubation atmosphere	Percentage of added activity recovered as $^{14}CO_2$
1	Oxygen	26
2	Nitrogen	Less than 0·2

Table 4. *Inhibition by cyanide of the conversion of formate to* CO_2 *in rat-liver homogenate*

(To 3 ml. samples of a supernatant, obtained by 15 min. centrifugation at 200 *g* of a homogenate (1 part of liver + 2 parts of medium) KCN was added to the final concentrations indicated. Incubated at 38° for 60 min. with 0·4 ml. solution containing 5 μmol. $H^{14}COONa$ with 25,600 counts/min. Incubation atmosphere: atmospheric air.)

Final KCN concentration (M)	0	3×10^{-3}	10^{-3}	3×10^{-4}	10^{-4}	3×10^{-5}
Percentage of added activity recovered as $^{14}CO_2$	58·5	2·9	17	48	56·5	58·5
Inhibition (percentage) of conversion	—	95	71	18	3	0

Table 5. *Influence of ageing, dialysis and* Co I *on the conversion of formate to* CO_2 *in a preparation of rat-liver homogenate*

(A supernatant was prepared from homogenate (1 part of liver + 3 parts of medium) by preliminary centrifugation for 15 min. at 5000 *g*, followed by two successive centrifugations, each for 30 min. at 20,000 *g*. The resulting final supernatant appeared practically particle-free on microscopy.

Part of this supernatant (*A*) was immediately used for an experiment, while another part (*B*) was kept for 6 hr. at 4° and a third part (*C*) dialysed against medium for 6 hr. at 4°. In all experiments 0·2 ml. of a solution containing 5 μmol. $H^{14}COONa$ with 25,600 counts/min. was added to each flask. Incubation: 60 min. at 38° in atmospheric air.)

	1	2	3	4	5
Supernatant and medium (*M*) used (ml.)	1·5 *A* + 1·5 *M*	1·5 *B* + 1·5 *M*	1·5 *C* + 1·5 *M*	1·5 *C* + 1·5 boiled *B*	1·5 *C* + 1·5 *M*
Further additions	0·2 ml. water	0·2 ml. water	0·2 ml. water	0·2 ml. water	2 mg. Co in 0·2 ml. water
Percentage of added activity recovered as $^{14}CO_2$	24·2	7·1	2·0	7·7	25·3

DISCUSSION

The fact that none of the activity added to a homogenate as labelled formate is incorporated into serine, while, as indicated by the simultaneous glycine incorporation, new formation of serine takes place at an appreciable rate, seems inconsistent with the idea of formate as the only immediate precursor of the β-carbon of serine. If formate really held this position such results might be obtained: (*a*) if the turnover of free formate was so extremely fast that its specific activity approached zero very soon after the beginning of the incubation, or (*b*) if the specific activity of the residual formate had approached zero towards the end of the incubation period and if, in addition, the turnover in the β-carbon of the free serine was very fast so that its activity would only mirror the activity in formate at the end of the incubation.

The following experimental observations, however, exclude these possibilities. Formate incorporation was lacking in cases where considerable formate activity was left in the homogenate at the end of the incubation, and its specific activity not demonstrably decreased (samples of a vacuum distillate, prepared according to Grant (1948), were examined for total formate activity by mercuric chloride degradation after addition of carrier formate and for total formate content, by Grant's colorimetric method). In Exp. 6 of Table 2, for example, 15 % of the activity added could be recovered as formate. If (*b*) took place, the use of an increased serine pool during the incubation should lead to greater trapping of formate activity in serine. This, however, did not occur, as is shown by the result of Exp. 8, Table 2, in which the addition of 15 mg. DL-serine before incubation caused no formate incorporation although the addition of serine (as indicated by the glycine incorporation) caused no decrease in the new formation of serine. Thus it appears that formate as such cannot be the only precursor of the β-carbon of serine.

It should be pointed out that several explanations could account for the fact that formate is not incorporated into serine, while glycine is. Thus if glycine and formate were incorporated by entirely independent processes, the one responsible for formate incorporation could be blocked in the homogenates. On the other hand, serine formation might actually be brought about by a coupling of glycine and a formate derivative. In this case such conversions of glycine which possibly were necessary before its coupling with a formate derivative would be produced in the homogenate, but the transformation of formate into its appropriate derivative would not be effected. Other carbons, possibly including the β-carbons from serine undergoing decomposition, would then serve as sources for the β-carbon of newly formed serine in homogenates. It is possible that formate is first reduced to formaldehyde and that this in turn combines with the glycine; and the enzyme system for conversion of formate to formaldehyde might be destroyed in homogenates.

In the case of the non-homogeneous system of slices suspended in medium there are of course several possible explanations why formate is incorporated to a lesser extent than glycine. This finding could however be satisfactorily explained by assuming, as suggested by the above discussion, that formate has to be converted into a derivative, also formed from other sources, before it can be incorporated into serine.

The occurrence in homogenates of serine synthesis without formate incorporation suggests the use of homogenates in testing whether other substances which are known to act as β-carbon sources *in vivo*, do so through the formation of formate or not. A few experiments were carried out with methyl-labelled choline instead of formate, but since in homogenates

only a questionably significant activity was found in the β-carbon of serine (while glycine was incorporated to a great extent), these experiments do not allow us to decide whether labile methyl groups may be transformed into the β-carbon by another route than via formate.

The oxidation of formate in homogenates and the simultaneous absence of formate incorporation into serine indicate that such an incorporation cannot be a necessary step in the oxidation of formate in the rat liver.

From the results obtained in the closer study of formate conversion to carbon dioxide in homogenate preparations (Tables 3–5) it seems likely that formate decomposition is caused by a non-particle-bound dehydrogenase, requiring Co I as a coenzyme and further dependent on the presence of a cyanide-sensitive electron mediator. Formate oxidation was also demonstrated in homogenates of rabbit liver and kidney cortex.

SUMMARY

1. In rat-liver slices formate carbon and the carbon of the labile methyl groups of choline are incorporated in the β-carbon of free serine.

2. Such incorporation could not be demonstrated in rat-liver homogenates even though the simultaneous incorporation of glycine indicated that new formation of serine took place.

3. Formate is oxidized to carbon dioxide in rat-liver homogenate; the enzyme system responsible for this process has been studied.

4. Based on these findings the conclusions are drawn that (*a*) formate is not the only (or is not the) immediate precursor of the β-carbon of serine, (*b*) the oxidation of formate in rat liver does not involve an incorporation into serine.

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The Vitamin B_1 Sparing Action of Fat and Protein

4. THE EFFECT OF CARBOHYDRATE IN DIETS DEFICIENT IN VITAMIN B_1 UPON THE SURVIVAL AND VITAMIN B_1 CONTENT OF THE RAT

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Previous papers in this series (Banerji, 1940, 1941) have shown that rats, given no vitamin B_1, can thrive provided that the diet is free from sucrose. Subsequently it was demonstrated that on diets devoid of the vitamin, irrespective of the presence or absence of sucrose and so irrespective of the development or otherwise of signs of deficiency, kidney slices of rats show a specific metabolic defect, defective oxidation of pyruvate *in vitro*, which is restored when the vitamin is added to the respiring slices (Banerji & Yudkin, 1942). From these results, the conclusion was drawn that signs of deficiency are not due to the inability *per se* to carry out an essential metabolic function—the proper metabolism of carbohydrate—but to an attempt to metabolize dietary carbohydrate in the absence of vitamin B_1. In other words, the animal can grow and thrive in the absence of vitamin B_1 provided it does not have to metabolize dietary carbohydrate. The signs of deficiency appearing when carbohydrate is fed would, on this hypothesis, be due to toxic products of incomplete or perverted carbohydrate metabolism.

Some of the consequences of this hypothesis have been examined in the work to be described. First, it was of interest to see whether and to what extent variation in the amount of dietary carbohydrate affected the rate of development of signs of deficiency. Especially it was interesting to see whether animals

were able to deal with small quantities of dietary carbohydrate in the absence of vitamin B_1. Secondly, an assessment was made of the amount of the vitamin in the tissues of animals with or without carbohydrate in the diet and receiving no vitamin B_1. Although the results of the experiments on respiration of tissue slices made it unlikely that there was any appreciable store of vitamin B_1 in the tissues, it is possible to explain the absence of signs in deficient animals receiving no carbohydrate by assuming a higher store of the vitamin in their tissues than in those of animals receiving carbohydrate. This might have arisen either by a decreased utilization of the vitamin when there was no dietary carbohydrate or by a synthesis of the vitamin in the alimentary canal on a carbohydrate-free diet.

EXPERIMENTAL

Animals. The rats used in all experiments were male piebald animals of the Hartwell strain, and experimental groups were constructed by adequate distribution of litter mates. The animals were kept in individual cages throughout the whole experiment which began when they were about 5 weeks old and weighed about 80 g.

Diets. The protein was light white casein (sodium caseinate, Glaxo Laboratories Ltd.); the fat was arachis oil when less than 40% was used and lard when a higher proportion was used. The carbohydrate was sucrose in all experiments, but in one of them glucose was also used. The protein, fat and carbohydrate made up 95 parts of the diet, to which were added 5 parts of salt mixture. Vitamins of the B group were supplied in the form of autoclaved marmite (12 parts to 100 parts of diet) and vitamins A and D in cod liver oil (2 drops daily to each rat).

Effect of amount of dietary carbohydrate on survival in the absence of vitamin B_1

Experiment 1. Twenty-four animals, in six groups of four, were placed on diets containing varying amounts of sucrose (Table 1). Diets 1, 2, 3 and 6 were of the same composition as those previously referred to as 'high-protein', 'high-fat', 'protein-fat', and 'standard basal' (Banerji & Yudkin, 1942).

Table 1. *Effect of variation in dietary sucrose on survival time*

(All diets contained 4·5% salt mixture and 10·5% autoclaved marmite. Cod liver oil, 2 drops, was given daily to each rat.)

Diet	Composition (%) Sucrose	Fat	Protein	No. of animals	Mean survival ±S.E. (days)
1	0	13	72	4	>140
2	0	67	18	4	>140
3	0	49	36	4	>140
4	18	31	36	4	46±3·0
5	36	22	27	4	33±0·5
6	54	13	18	4	31±0·7

All the animals on the first three (carbohydrate-free) diets survived in apparent health for 5 months, after which they were killed. Those receiving 18% sugar survived appreciably longer than those receiving 36 or 54% sugar.

Experiment 2. Five groups, each of four animals, were placed either on the high-protein diet, or on diets containing varying amounts of sucrose; in this experiment, the proportion of fat in the diet was constant. One of the animals on the high-protein diet died after 95 days; the others were apparently healthy when killed after 112 days. The animals on the other diets survived for the longest time with a diet containing about 9% sugar and for shorter times with increasing amounts of sucrose (Table 2).

Table 2. *Effect of variation in dietary sucrose on survival time*

(Proportion of dietary fat constant. For further details, see Table 1.)

Diet	Composition (%) Sucrose	Fat	Protein	No. of animals	Mean survival ±S.E. (days)
1	0	13	72	4	>108
7	9	13	63	4	78±4·2
8	18	13	54	4	55±3·2
9	36	13	36	4	33±2·6
6	54	13	18	4	43±1·8

Experiment 3. In this experiment, the proportion of dietary protein was constant and the proportion of sucrose and fat varied. Again, there were four animals in each group. One animal on the high-fat (sucrose-free) diet died after 87 days; the other three survived until they were killed after 122 days. The survival of the rats on the diets containing sucrose decreased, as in Exp. 2, as the amount of sucrose increased (Table 3).

Table 3. *Effect of variation in dietary sucrose on survival time*

(Proportion of dietary protein constant. For further details, see Table 1.)

Diet	Composition (%) Sucrose	Fat	Protein	No. of animals	Mean survival ±S.E. (days)
2	0	67	18	4	>114
10	9	58	18	4	63±2·9
11	18	49	18	4	50±2·1
12	36	31	18	4	35±2·2
13	45	22	18	4	35±1·9
6	54	13	18	4	32±2·3

Experiment 4. It is hoped to study in detail the comparative effects of different carbohydrates on the development of signs of deficiency of vitamin B_1. One such experiment has been carried out, in which glucose was used instead of sucrose. The results were similar in that increasing amounts of either sugar

Table 4. *Effect of variation in dietary sugar on survival time*

(Proportion of dietary fat constant.)

Diet	Composition (%) Sucrose	Glucose	Fat	Protein	No. of animals	Mean survival ± S.E. (days)
7	9	0	13	63	4	65 ± 2·4
7G	0	9	13	63	4	83 ± 1·0
8	18	0	13	54	4	46 ± 1·5
8G	0	18	13	54	4	63 ± 4·0
9	36	0	13	36	4	35 ± 1·7
9G	0	36	13	36	4	53 ± 2·7

Table 5. *Effect of sucrose on vitamin B_1 content of tissues*

Diet	Daily supplement vitamin B_1 (μg.)	No. of animals	Vitamin B_1 (μg./g.) in Brain	Liver	Kidney	Muscle
Standard basal	0	6	0·74 ± 0·049	0·43 ± 0·038	0·51 ± 0·040	0·26 ± 0·024
High-protein	0	6	0·81 ± 0·050	0·50 ± 0·041	0·45 ± 0·037	0·22 ± 0·020
High-fat	0	6	0·73 ± 0·045	0·41 ± 0·032	0·48 ± 0·032	0·25 ± 0·021
Standard basal	10	6	2·3 ± 0·22	2·9 ± 0·31	2·3 ± 0·29	1·0 ± 0·09
High-protein	10	6	2·3 ± 0·25	2·7 ± 0·30	2·0 ± 0·20	1·0 ± 0·10
High-fat	10	6	2·1 ± 0·25	3·0 ± 0·29	2·1 ± 0·18	1·1 ± 0·09
Standard basal	4	4	—	0·46 ± 0·41	—	0·24 ± 0·022
Standard basal	8	6	—	2·9 ± 0·30	—	0·65 ± 0·044
Standard basal	27	4	—	4·8 ± 0·42	—	1·1 ± 0·11
Standard basal	40	5	—	6·2 ± 0·54	—	1·15 ± 0·12

lessened the times of survival, but survival was somewhat longer on glucose than on sucrose (Table 4).

Effect of sucrose upon the content of vitamin B_1 in the tissues

Experiment 5. Thirty-six rats were used in this experiment. They were given one of three diets; the high-protein diet (1), the high-fat diet (2), or the standard basal diet containing sucrose (6). Six animals on each diet were given no supplement and the other six were each given 10 μg. vitamin B_1 daily. The animals on the standard basal diet with no supplement developed severe deficiency after about 4 weeks and each was killed a day or two before it would have died. The remainder grew well and were killed after 5 months.

Vitamin B_1 was estimated in the brain, liver, kidney and skeletal muscle by the thiochrome method of Wang & Harris (1941). The whole brain, both kidneys and samples of about 2 g. liver and thigh muscle were used.

In the results given in Table 5, figures are also added for analyses done on liver and muscle in the course of other work with rats receiving the standard basal diet and 4, 8, 27 or 40 μg. vitamin B_1 daily (Yudkin, 1949). The animals receiving 4 μg. were killed when moribund and the others after 4 or 5 months.

It will be seen that the presence of sucrose in the diet did not affect the amount of vitamin B_1 remaining in the tissues. More particularly, animals receiving no vitamin B_1 in the diet had similar stores of the vitamin, whether or not the diet contained sucrose and whether or not the animals developed signs of deficiency.

DISCUSSION

The results of these experiments support the view that animals are able to thrive with no vitamin B_1 provided their diets contain no carbohydrate. With smaller amounts of dietary carbohydrate they are able to survive longer than with larger amounts, but they succumb when the diets contain as little as 9% sucrose or glucose. The stores of vitamin in animals receiving no sucrose are as low as in those receiving sucrose and consequently dying of deficiency. The survival of the former, therefore, is due to an ability to carry out their essential metabolic functions in the absence of vitamin B_1, although this ability does not extend to the proper metabolism of dietary carbohydrate. When, therefore, carbohydrate is fed without the vitamin, an abnormal metabolism of carbohydrate occurs and this is responsible for the sign of deficiency.

In a recent publication, Gruber (1950) has attempted to distinguish between two meanings of the phrase 'vitamin sparing' as used to describe the effect of increased dietary fat in delaying the onset of signs of deficiency of vitamin B_1. He says, 'The verb "to spare" can have a double meaning here, namely,

"to dispense with" or "to use less of"....According to the first meaning, an increased carbohydrate ingestion would provoke symptoms of aneurin deficiency at a level of this vitamin in the tissues at which these symptoms would not yet appear with lessened carbohydrate intake. According to the second meaning, the increased consumption would entail a bigger loss of aneurin from the tissues.' He estimated vitamin B_1 in the tissues of pigeons fed for 13 days on three diets, containing about 90, 70 and 0% of sucrose, and found increasing amounts of the vitamin with decreasing amounts of dietary sucrose. These results he interprets as showing that the sparing action of fat means that the fat decreases the use of the vitamin, rather than that the fat allows the animal to dispense with it.

The work reported in this and the previous paper, however, make it clear that fat (and protein) are able to spare vitamin B_1 in both senses; not only do animals use less vitamin B_1 with decrease in dietary carbohydrate, but in the complete absence of dietary carbohydrate they are, in fact, able to dispense with it altogether. The effect of sparing is, therefore, quantitative in the sense that the animals use the vitamin less rapidly, and so survive longer, when the proportion of dietary carbohydrate is decreased. But it later becomes qualitative, in the sense that the small amount of vitamin B_1 remaining in the tissues after a shorter or longer period of deprivation is adequate for survival in the absence of dietary carbohydrate, but inadequate in its presence.

However, even with no carbohydrate in the diet, the animals are still presumably metabolizing carbohydrate, produced from the metabolism of both fat and protein. It is possible that the animal has, in fact, two pathways for the metabolism of carbohydrate, one involving vitamin B_1 and the other not. The latter must then be considered to be limited, and sufficient only to deal with an amount of carbohydrate such as that produced from fat and protein. Alternatively, it is possible that the organism deals differently with carbohydrate derived from fat or protein, and carbohydrate taken in the diet. Further, it may be that, with no dietary carbohydrate, the consumption of larger amounts of fat and protein increases, by a process of adaptation, the utilization of these substances without the intermediate formation of carbohydrate. The observations of Gilmore & Samuels (1949) are of interest in this connexion; they showed that the diaphragm of the rat after a high-fat diet utilizes *in vitro* far less carbohydrate than one from an animal fed carbohydrate. More recently, Wertheimer & Ben-Tov (1950) have demonstrated adaptive changes in the metabolism of carbohydrate in rats which have been given a restricted diet. There was an increased storage of glycogen in the liver and in the muscles and a deposition of glycogen in the adipose tissue; the latter is taken as indicating an increased conversion of carbohydrate to fat.

Further work is now in progress to study the metabolism of carbohydrate, fat and protein in animals on carbohydrate-free diets.

SUMMARY

1. Rats consuming as little as 9% sucrose or glucose in their diets were not able to survive in the absence of vitamin B_1 as did those receiving no carbohydrate. They did, however, survive longer than those consuming diets with higher proportions of carbohydrate.

2. The amount of vitamin B_1 in the tissue of animals receiving no vitamin B_1 was unaffected by the presence or absence of carbohydrate in the diet, even though those consuming carbohydrate were moribund and the others were well.

3. The results support the hypothesis that signs of deficiency are due to the disturbed metabolism of carbohydrate in the absence of vitamin B_1.

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Mode of Formation of Milk Fatty Acids from Acetate in the Goat

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In a previous paper (Popják, French & Folley, 1951) some results of an experiment carried out on a lactating goat with the aid of $CH_3{}^{14}CO_2Na$ were described. It was found that, in the origin of milk fat, synthesis within the udder from acetate played an outstanding part. This conclusion was based on a comparison of the specific activities of four crude fatty-acid fractions obtained from milk glycerides with those of the plasma fatty acids after a single injection of $CH_3{}^{14}CO_2Na$.

During the first 12 hr. of the experiment the steam-volatile acids insoluble in water (consisting mainly of C_8–C_{12} acids) had the highest specific activities, the next in order being the water-soluble steam-volatile acids (C_4–C_8 acids). These were followed by the specific activity of the non-volatile solid and liquid acids, the latter having the lowest specific activities. From 12 hr. onwards, however, the specific activity-time curve of the non-volatile solid acids gradually crossed over the specific activity-time curves of the two steam-volatile fractions so that by the 23rd hour of the experiment the specific activities of the four fractions were in the following order: non-volatile solid acids > water-insoluble steam-volatile acids > water-soluble steam-volatile acids > non-volatile liquid acids. This same order was maintained until the end of the experiment (48 hr.) (cf. Popják *et al.* 1951, Fig. 3).

The specific activity-time curves of all four fractions, after reaching their maxima 3–4 hr. after the injection of labelled acetate, decayed smoothly with an average half-life of about 4 hr. The similarity of the half-lives suggested that the different specific activities of the fractions were in some way connected with the biochemical mechanism of fatty-acid synthesis rather than with any other factor (e.g. differences in turnover time, dilution with non-isotopic blood fat). Since the specific activities of these mixtures of fatty-acid samples bore no simple relationship to the average chain length of their component acids, it was pointed out that resolution into individual acids was required in order to interpret more fully the role of acetate in milk-fat synthesis.

In this paper the results of complete fractionations of the fatty-acid samples obtained in the previously described experiment are reported together with the chemical degradations of acetic, butyric and caproic acids, in which the position of the ^{14}C-label in individual carbon atoms of the fatty-acid chain has been established.

It would have been a gargantuan task to resolve into individual acids the four crude fatty-acid fractions from twenty-two milk samples. If the specific activities of the four fractions had maintained the same order relative to one another throughout the experiment, it would have been justifiable to pool the samples from the entire experiment for the isolation of the individual acids. However, because of the change in the relative position of the specific activity-time curves at 12 hr. after the injection, it was decided to divide the acids into two main groups, (1) those obtained up to 12 hr. and (2) those between 12 and 48 hr. of the experiment. Thus during the first period the fatty acids had their highest specific activities and during the second period the specific activities of all the fractions were already declining. In addition to these two main parts of the experiment, some results obtained on two small samples of the water-soluble volatile acids, one representing 0–7 hr. and the other approximately the entire experimental period, are also presented.

It is now apparent that, although acetate is a major source of carbon for milk fatty-acid synthesis, the results strongly suggest that another precursor, which we believe to be a C_4 compound, derived from the blood, must also be involved. As a result of the participation of this second precursor of low isotope content, it has been possible to show by chemical degradations that the synthesis of fatty acids must occur by the stepwise elongation of a shorter acid at the carboxyl end with a C_2 compound and not by the simultaneous condensation of C_2 units. Some of these results have been published in a preliminary form (French, Hunter, Martin & Popják, 1951).

RESULTS

Table 1 shows the extent of fractionation achieved. All the acids, with the exception of acetic, were chromatographically pure. The acetic acid was contaminated with probably not more than 10% of butyric acid, but as the quantities of this acid were rather small (5–10 mg.) no further purification was attempted.

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Table 1 shows that there is a definite trend in the specific activities of the pure acids with increasing chain length from butyric acid upwards. During the first 12 hr. of the experiment the specific activities increased up to and including capric (decanoic) acid, whereas during the second part of the experiment the specific activities increased up to myristic (tetradecanoic) and palmitic (hexadecanoic) acids.

Table 1. *Specific activities* (1×10^{-3} μc. ^{14}C/mg. C) *of pure fatty acids obtained from glycerides of milk during various intervals after the intravenous injection of 5 mc. of* ^{14}C *as* $CH_3{}^{14}CO_2Na$ *into a lactating goat*

Fatty acid	Period of experiment from which fatty acids were pooled			
	0–7 hr.	0–48 hr.	0–12 hr.	12–48 hr.
Acetic	—	11·80	14·68	—
Butyric	18·00	7·63	11·45	0·350
Caproic (hexanoic)	28·75	9·86	21·15	0·424
Caprylic (octanoic)	—	—	28·40	0·717
Capric (decanoic)	—	—	33·75	0·943
Lauric	—	—	23·80	1·080
Myristic	—	—	10·28	1·260
Palmitic	—	—	10·25	1·260
Stearic	—	—	0·49	0·063
Oleic	—	—	0·31	0·063

This difference between the two parts of the experiment explains the crossing over of the specific activity-time curves of the steam-volatile acids and the solid non-volatile acids (Popják *et al.* 1951, Fig. 3).

The meaning of these results will be discussed below under individual acids, together with the information obtained from the chemical degradation of acetic, butyric and caproic acids, which are given in Table 2.

Acetic, propionic and valeric acids

By using standard methods of fat extraction, saponification, extraction of the unsaponifiable material from the soaps followed by steam distillation, we consistently demonstrated the presence of acetic acid among the water-soluble steam-volatile fatty acids by paper chromatography. It amounted to 1–2% of the total water-soluble steam-volatile acids. Two of these samples were isolated as the silver salt for radioactive assay. It is impossible to say at present whether the acetic acid was present originally as glyceride or combined with another fat-soluble compound. Its relationship to the metabolically active acetate used in the synthesis of the higher fatty acids is therefore not clear and its relatively low specific activity cannot be accounted for. The possibility has to be considered also that the acetic acid might have been derived partly by bacterial decomposition of milk constituents during the period between milking and extraction of the fat. The further possibility that the presence of acetate in the milk fat was an artifact due to temporary raising of the blood acetate level by the injection of $CH_3{}^{14}CO_2Na$ may be dismissed, since acetate was found in the milk at all times of the experiment up to 48 hr. after the injection. Whatever the origin of this acetate, its occurrence nevertheless served as a useful guide showing that redistribution of the label from the injected acetate did not occur in the animal (cf. Table 2) since ^{14}C was found only in the carboxyl carbon. In the subsequent discussion of fatty-acid synthesis from the injected acetate, we shall refer to 'acetate' to denote the metabolically active C_2 derivative of acetic acid. There is no evidence available from our experiment to show what this active derivative might be, nevertheless it appears justifiable to speak of the methyl and carboxyl carbon atoms of this metabolite, since from evidence discussed below it is clear that the polarity of the molecule was maintained.

In addition to acetic acid, propionic and valeric acids were also found among the water-soluble steam-volatile fatty acids by paper chromatography. No radioactivity could be detected over the spots of these acids on the paper although the activities of acetic, butyric and caproic acids were definitely measurable on the paper. The considerations as to the origin of acetic acid apply also to the origin of propionic and valeric acids. Both of these acids, however, are known to occur in the rumen and therefore may find their way into the milk via the blood. In this connexion it might be mentioned that in an as yet unpublished experiment, in collaboration with Prof. G. Peeters and Dr S. J. Folley, in which an isolated cow udder was perfused with blood containing acetate, no propionic or valeric acid was found in the milk fat.

Butyric acid

The butyric acid had a lower specific activity in both periods of the experiment than any longer acid up to and including lauric (dodecanoic); later its ^{14}C content was the lowest of all acids up to palmitic (hexadecanoic) (Table 1). The degradation of butyric acid revealed the presence of equal amounts of ^{14}C in carbon atoms 1(COOH) and 3, carbons 2 and 4 being totally inactive. This finding indicates that butyric acid has been synthesized in the udder by the condensation of two acetate units of equal isotope content, the methyl carbon of one having been linked with the carboxyl carbon of the other. It has been mentioned in the introduction that we do not consider acetate to be the only source of butyric acid in the milk. The necessity for this assumption should become clear from the information obtained by the degradation of caproic acid.

Table 2. *Specific activity* (1×10^{-3} μc. ^{14}C/mg. C) *of individual carbon atoms in acetic, butyric and caproic acids from milk glycerides after intravenous injection of 5 mc. of* $CH_3{}^{14}CO_2Na$

Period of experiment (hr.)	Fatty acid	C atom numbers 6	5	4	3	2	1(COOH)	Average of all C atoms
0– 7	Butyric	—	—	0·00	36·00	0·00	36·00	18·00
	Caproic	0·00	36·20	0·00	36·20	0·00	96·70	28·75
0–48	Acetic	—	—	—	—	0·00	23·60	11·80
	Butyric	—	—	0·00	15·26	0·00	15·26	7·63
	Caproic	0·00	13·20	0·00	13·20	0·00	32·60	9·86
12–48	Butyric	—	—	0·00	0·700	0·00	0·700	0·350
	Caproic	0·00	0·556	0·00	0·556	0·00	1·504	0·424

Caproic (hexanoic) acid

The results of the degradation of caproic acid from the early part of the experiment (cf. Table 2) should be considered first. Only carbon atoms 1(COOH), 3 and 5 contained ^{14}C. The specific activity of the COOH carbon was about 2·5 times that of carbons 3 and 5 whose specific activities were equal, just as in carbons 1 and 3 of butyric acid. Further, the specific activities of carbons 3–6 of caproic acid were sufficiently close to the activities of the butyric acid carbons as to leave very little doubt that in fact these carbons were derived from the carbon chain of butyric acid. Therefore the synthesis of caproic acid must have occurred by the elongation of this shorter chain, at what corresponds to the carboxyl carbon of butyric acid, by the addition of acetate. It may be inferred from the similarity of the radioactivities of carbons 3–6 of caproic to those of butyric acid that the newly synthesized caproic acid has not been diluted in the udder to a significant extent by pre-existing non-isotopic caproic acid, i.e. the metabolic 'pool' of caproic acid in the gland must be very small.

The difference between the ^{14}C content of the 'acetic' and 'butyric' parts of caproic acid would admit at first sight two possible explanations. The first one is that the butyric acid synthesized from acetate was diluted 2·5 times by pre-existing non-isotopic butyrate. This would imply a fairly large pool of this acid. If this were the case, then during the second part of the experiment, when the specific activity of acetate must have been declining rapidly, the 'acetic' part of caproic acid should have a lower specific activity than the 'butyric' part. The second possible explanation is that the butyric acid synthesized from acetate is being continuously diluted 2·5 times by a non-isotopic C_4 compound derived from the blood. This C_4 compound might be butyric acid itself or another C_4 compound which is converted into the acid. If this second explanation is the right one, the 'butyric' part of caproic acid in the milk should have a lower isotope content than the 'acetic' part at all times of the experiment.

The degradation of caproic acid from the later part of the experiment showed that even here the specific activity of its 'acetic' part was about 2·5 times greater than that of its 'butyric' part. Therefore the contribution of the first mechanism to explain the observed differences must be negligible. There is a good deal of evidence to show that the pools of the volatile acids up to capric (decanoic) (see later) within the cells of the mammary gland are very small.

We cannot avoid, therefore, the conclusion that the butyric acid synthesized from acetate in the udder is continuously being diluted about 2·5 times by a non-isotopic C_4 compound. Thus butyrate in the udder seems to have a dual origin which may be represented as follows:

$$\left.\begin{array}{l} 2CH_3C^{**}OOH \rightarrow CH_3C^{**}H_2CH_2C^{**}OOH \\ C_4 \text{ compound (non-isotopic} \rightarrow CH_3CH_2CH_2COOH \end{array}\right\} = CH_3C^{*}H_2CH_2C^{*}OOH.$$

The synthesis of caproic acid may then be visualized by

$$CH_3C^{*}H_2CH_2C^{*}OOH + CH_3C^{**}OOH \rightarrow CH_3C^{*}H_2CH_2C^{*}H_2CH_2C^{**}OOH.$$

Caprylic (octanoic) and capric (decanoic) acids

The increase of specific activity with increasing chain length is also observed in the case of caprylic (octanoic) and capric (decanoic) acids at all times of the experiment (cf. Table 1). These acids have not been degraded yet, but the results are consistent with the view that they are being synthesized by the same mechanism as outlined for caproic acid, i.e. caprylic by the elongation of caproic and capric by the lengthening of caprylic acid. Also the pools of these acids presumably must be small, otherwise the newly synthesized acids of high ^{14}C content would have been diluted by pre-existing non-isotopic acids and the increasing specific activities with increasing chain lengths could not have been observed during the entire experimental period.

Lauric (dodecanoic) acid

During the first 12 hr. lauric (dodecanoic) acid had a lower activity than capric or caprylic. Later, however, its activity became higher than that of capric. That lauric acid is synthesized by the same mechanism as for the shorter acids appears probable from the position which it occupies during the second period. However, since in the earlier period lauric acid does not attain as high activity as capric acid, its turnover time must be more prolonged than that of the shorter acids. It is probable that the cells contain an appreciable pool of lauric acid.

Myristic (tetradecanoic) and palmitic (hexadecanoic) acids

These acids have equal activities in both the early and late periods, but relative to the other acids they change from a low level to become eventually the most active of the acids. The arguments adduced in the case of lauric acid could apply to these two also, since it is likely that these acids, which are regular components of tissue fat, are present in considerable quantity within the cells. The equal activity of myristic and palmitic acids may suggest that the two acids are in equilibrium, i.e. that palmitic is broken down to myristic as well as being formed from it.

Stearic (octadecanoic) and oleic (octadec-9-enoic) acids

The very low relative activity of these acids (Table 1) seems to suggest that they have a different mode of origin, being derived largely from preformed molecules in the blood, or synthesized chiefly from a precursor other than acetate. However, it is possible that relatively large pools of these acids in the cells play a part in producing the observed low activities, for by the later part of the experiment a relative increase in these activities has occurred.

In the first period stearic acid was slightly more active than oleic, but later the activities became equal. This suggests a possible equilibrium between the two acids.

DISCUSSION

Certain general principles of fat metabolism and milk secretion are implicit in the present results. It has been stated in the previous paper (Popják *et al.* 1951) that the formation of milk-fat results from the endogenous metabolism of the gland cells and that fatty acids taken up from the blood probably are broken down more or less to C_2 units which become mixed with exogenous acetate. From the results presented above the further points which have emerged are: (1) the stepwise elongation of fatty-acid chains at the carboxyl end by the addition of a C_2 unit derived from acetate; (2) the participation of a C_4 compound in the formation of butyric acid; and (3) the small size of the metabolic pools of the short-chain acids (butyric-capric) in the mammary gland cells.

It is possible on the basis of these results, to suggest the main features of the synthetic process, and to consider whether this is peculiar to the mammary gland or whether it is a general process in mammalian tissue. The question is, if body fat and milk fat are synthesized from the same precursor(s), why do the products differ in composition?

Acetate is known to be a building-stone available for all positions of the fatty-acid chain (see Bloch, 1947, 1948). The interconversion of palmitic and stearic acids by the addition or removal of C_2 units was demonstrated by the experiments of Schoenheimer & Rittenberg (1937) and of Stetten & Schoenheimer (1940) with deuterium-labelled fatty acids. Zabin (1950), in Dr K. Bloch's laboratory, has recently shown in rats, under conditions in which fatty-acid synthesis is depressed, that after administration of $CH_3{}^{14}CO_2Na$, almost all the radioactivity of stearic acid could be accounted for by the radioactivity of the carboxyl carbon. The elongation of lauric (dodecanoic) and myristic (tetradecanoic) acids to palmitic and stearic acids has been shown to occur also (Klem, 1943). Thus the elongation of fatty acids by the addition of a C_2 unit is well established for the longer acids which are constituents of body fats.

The short-chain acids, which were shown in this investigation to be formed likewise by the lengthening of a still shorter acid by the addition of an acetate molecule and which might be expected as intermediates in the total synthesis of the longer ones, are, however, thought to be absent from cells of internal organs. Moreover, Rittenberg, Schoenheimer & Evans (1937) failed to find any evidence that deuterium-labelled butyric and caproic acids could be used for synthesis of higher acids. Therefore, a scheme of simple reversal of β-oxidation as a general mechanism of fatty-acid synthesis in the tissues (with the exception of the mammary gland as presented here) could not be supported by experimental evidence. An alternative hypothesis of simultaneous condensation of C_2 units, giving long-chain acids directly, has been considered (e.g. by Peters & Van Slyke, 1946). It should, however, be pointed out that the failure of the deuterium-labelled butyric and caproic acids to give rise to fatty acids labelled with deuterium does not constitute an absolute proof against the possibility of the carbon chain of these acids having been utilized in the synthesis of higher acids.

The question of the general applicability of the mechanism of fatty-acid synthesis as revealed in the mammary gland hinges on the relationship which exists between the fatty acids found in the milk triglycerides and the various precursors which are

undergoing metabolism within the cells. This relationship is a matter of conjecture as far as the chemical form in which the precursors react is concerned, but it can be concluded from our results that the carbon chains take part in the synthesis in the manner outlined above. The glyceride fatty acids of the milk are perhaps best regarded as metabolic products which have become stabilized by esterification with glycerol and secretion into the milk ducts. The process of secretion might be regarded as one which intervenes with great rapidity between the formation of these acids and their further metabolism in the cell, so as to trap in the milk glycerides substances which would otherwise be metabolized so rapidly as never to accumulate to any great extent. According to this argument then the difference between milk- and body-fat formation might be more apparent than real; the process of rapid secretion by the mammary gland having only the effect of 'preserving'—rather conveniently—the intermediary products of synthesis.

Stadtman, Stadtman & Barker (1949) have shown by the use of ^{14}C that *Clostridium kluyveri* synthesizes caproic acid by the addition of a C_2 unit derived from ethanol to the carboxyl carbon of butyric acid. Stadtman & Barker (1949*a–e*) have studied the reactions involved in this synthesis in considerable detail by means of a cell-free enzyme system. The anaerobic synthesis of fatty acids by this organism is essentially an energy-yielding mechanism, and it is not clear at present how far it is comparable to fat synthesis in animals. It is quite probable, however, that many of the intermediate steps are similar.

Smith & Dastur (1938) were the first to suggest that the short-chain fatty acids in the milk might be intermediates in the origin of the higher ones. Similar views, based on the *in vitro* utilization of specific substrates by mammary tissue with high respiratory quotients, have been expressed by Folley & French (1948, 1950). The results described in the present paper are, however, the first direct evidence on the question.

The participation of a C_4 precursor in the formation of milk fatty acids may be related to information available on the metabolism of lactating ruminants. Butyric acid itself is absorbed from the rumen, but its concentration in the peripheral blood is low (McClymont, 1949). The ketone bodies, however, circulate in considerable amount, and Shaw & Knodt (1941) have demonstrated the uptake of β-hydroxybutyrate by the udder of the lactating cow. They estimated the quantity taken up to be equivalent to all the milk acids up to and including tetradecanoic, i.e. more than one-third of the total acids. If this substance is used for milk-fat synthesis, it seems probable from our results, that it contributes to the formation of butyric acid and therefore also to the equivalent parts of the carbon chain of all acids up to palmitic. The quantitative extent of its contribution may thus be considerable, although it seems likely that some of it would be metabolized via a C_2 unit and thus become merged with the metabolic acetate.

The conception of milk-fat formation advanced here is somewhat at variance with that of Hilditch (1947) whose views have recently been re-affirmed (Achaya & Hilditch, 1950). It hardly need be stressed here that our results do not admit the possibility that the short-chain volatile acids in the milk were derived directly from the degradation of oleic acid, which according to Hilditch should be their primary source. We have not been able to isolate from our small material Δ^9 unsaturated acids of shorter chain length than oleic, which according to Hilditch should also be derived from oleic acid degradation, and therefore cannot offer direct evidence on their mode of origin. It should, however, be pointed out that the derivation of oleic acid from stearic and of palmitoleic by the dehydrogenation of palmitic acid have been proved (Stetten & Schoenheimer, 1940). It does not seem at all improbable, therefore, that tetradec-9-enoic, dodec-9-enoic and dec-9-enoic acids originate also from the dehydrogenation of the corresponding saturated acids.

EXPERIMENTAL

The crude fatty-acid fractions as described in the earlier publication (Popják *et al.* 1951) formed the starting material of the fractionations. These fractions were: (1) water-soluble and (2) water-insoluble steam-volatile acids; (3) non-volatile solid and (4) non-volatile liquid acids.

Methods of fractionation

Water-soluble steam-volatile fatty acids. These were resolved into individual acids by azeotropic distillation as described by Schicktanz, Steele & Blaisdell (1940). Benzene, toluene and xylene were used successively to separate acetic, butyric and caproic acids. The residual fatty acid, after the distillation with xylene was completed, was pure caprylic (octanoic) acid.

Water-insoluble steam-volatile fatty acids. These were separated by fractional distillation of the free acids at 6 mm. of Hg pressure in the distillation apparatus described by Schoenheimer & Rittenberg (1937). The capacity of our still was 5 g. Fractionation by partition chromatography (Howard & Martin, 1950) was also employed.

Non-volatile solid and liquid fatty acids. These were converted into methyl esters either by refluxing with dry methanol in the presence of H_2SO_4 or by treatment of the acids with diazomethane. The methyl esters were then distilled at 0·5 mm. of Hg pressure in the apparatus used for the fractionation of the water-insoluble volatile acids.

Identification of the fatty acids and criteria of their purity

Mostly chromatographic methods were used for this purpose: paper chromatography for acids up to and including caproic (hexanoic) (Martin, 1950) and partition

chromatography for acids of longer chain length (Howard & Martin, 1950). In addition, in the case of acids of C_{14} to C_{18} chain length (of which larger amounts were available), an essential criterion of purity was, before testing by chromatography, that the specific activity of the fraction should remain constant after redistillation.

The fatty acids, the specific activities and degradations of which are reported were pure chromatographically unless specified.

Methods of chemical degradations of fatty acids

Acetic acid was degraded by the pyrolysis of the Li salt *in vacuo* at 380°, essentially as described for barium acetate (see Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). The acetone formed during the pyrolysis was collected in a trap cooled with liquid air and subjected to the iodoform reaction. The carbon atom of CHI_3 corresponds to the methyl carbon of acetate. The Li_2CO_3, the other product of pyrolysis, the carbon atom of which is derived from the carboxyl carbon of acetate, was converted into $BaCO_3$ and used for the assay of ^{14}C.

Butyric acid was degraded by oxidation of ammonium butyrate with H_2O_2 as described by Wood, Brown, Werkman & Stuckwisch (1944). This degradation can be carried out conveniently on 200 mg. of butyric acid. The only product of the oxidation, useful for the identification of the labels in the carbon chain of butyric acid, is acetone, the carbonyl carbon of which is derived from carbon atom 3 and the methyl carbons from carbons 2 and 4 of butyric acid. The following equations represent the main reactions in this degradation:

$$\overset{4}{CH_3}.\overset{3}{CH_2}.\overset{2}{CH_2}.\overset{1}{COOH} \xrightarrow{H_2O_2} \overset{4}{CH_3}.\overset{3}{CO}.\overset{2}{CH_3} + CO_2 \quad (1)$$

$$\overset{4}{CH_3}.\overset{3}{CO}.\overset{2}{CH_3} \xrightarrow{NaOI} \overset{2,4}{CHI_3} + \overset{2,4}{CH_3}.\overset{3}{COOH} \quad (2)$$

Unfortunately the CO_2 evolved in reaction (1) is almost equally derived from all four carbon atoms of butyric acid.

The acetic acid, resulting from the iodoform reaction (2), was separated from the reaction mixture by steam distillation after removal of the excess I_2 with Ag_2SO_4. It was finally obtained as the Ag salt and its radioactivity determined. The radioactivity of the carboxyl carbon of butyric acid was obtained by difference.

Caproic acid was degraded by a new method (Hunter & Popják, 1951), according to which carbon atoms 1(COOH) and 2 of caproic acid are split off as acetic acid and carbons 3, 4, 5 and 6 give butyric acid. The steps involved in this degradation are: α-bromination of the acid chloride, conversion into ethyl or higher ester (best yield with *neo*pentyl ester), dehydrobromination of the α-bromo ester, yielding the ester of hex-2-enoic acid. The fusion of this ester with KOH at 330° results in the fission of the double bond and gives acetic and butyric acids which were degraded further by the methods already mentioned.

Assay of ^{14}C was carried out mostly on solid samples of 'infinite' thickness >25 mg./sq.cm. as described previously (Popják, 1950), but in a few instances the measurements were made on samples of 1–3 mg./sq.cm. thickness. The radioactive counts were all corrected to infinite thickness by the use of an experimentally determined self-absorption curve. The specific activities expressed in this paper as $\mu c.$ ^{14}C/mg. C may be converted into counts/min. by the following factors: a substance containing 1×10^{-3} $\mu c.$/mg. gave 1300 counts/min. in samples of 1 sq.cm. area or 2470 counts/min. in samples of 2 sq.cm. area in infinite thickness. Because of the high activity of our material the assays could easily be carried out to an accuracy of ±1% or better.

SUMMARY

1. Milk fatty acids obtained from a lactating goat after the injection of $CH_3{}^{14}CO_2Na$ have been resolved into individual acids and their ^{14}C content determined. All the even numbered saturated acids from C_2 to C_{18} and also oleic acid have been isolated. Traces of propionic and valeric acids were also identified by paper chromatography.

2. Chemical degradations of acetic, butyric and caproic acids have been carried out. Only the carboxyl carbon of acetic acid contained ^{14}C.

3. Butyric acid is synthesized from two acetate molecules in such a way that the methyl carbon of one becomes linked to the carboxyl carbon of the other. It is inferred that only about 40% of the butyric acid is synthesized from acetate, the other 60% being derived from a non-isotopic C_4 compound, possibly β-hydroxybutyric acid. Caproic acid is synthesized by the elongation of the butyric acid chain at the carboxyl end by the addition of acetate.

4. The metabolic 'pools' of the short chain acids (up to C_{16}) in the mammary gland cells appear to be very small.

5. The results are consistent with the view that all the milk fatty acids up to and including palmitic are formed by the stepwise elongation of a shorter acid by the addition of a C_2 compound derived from acetate. Stearic and oleic acids might be derived mainly from the blood or from a precursor other than acetate.

6. The possible relationship between the mechanism of fatty-acid synthesis in the mammary gland and in other mammalian tissues is discussed.

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Formation of Cellulose by Certain Species of *Acetobacter*

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Brown (1886) showed that the surface membranes (often designated pellicles) formed by *Bacterium xylinum* when grown on suitable carbohydrate media consist of cellulose, and this material has been examined by Hibbert & Barsha (1931), Tarr & Hibbert (1934), Khouvine, Champetier & Sutra (1932), Sisson (1936), Franz & Schiebold (1943), Frey-Wyssling & Mühlethaler (1946) and by Hestrin, Ashner & Mager (1947). *Bact. xylinum* is now known as *Acetobacter xylinum*. Frey-Wyssling & Mühlethaler (1946) have shown also that the pellicle formed by *Acetobacter xylinoides* Henneberg from glucose consists of cellulose, but the surface covers which are formed from certain substrates by *A. pasteurianum* Hansen, *A. kützingianum* Hansen and *A. acetigenum* Henneberg, have not hitherto been subjected to detailed examination.

In this laboratory, while studying bacteria from an East African vinegar brewery, Dr D. Kulka isolated a strain of *A. acetigenum* (hereafter referred to as *A. acetigenum* E.A.) which produced very thick pellicles on glucose media. Chemical examination showed the pellicle material consisted of cellulose and this was confirmed by X-ray examination. The results of the X-ray examination are reserved for a future communication.

The production of cellulose by this organism, by *A. acetigenum* (type culture), by *A. pasteurianum* and by *A. kützingianum*, is now described.

EXPERIMENTAL AND RESULTS

Cultural conditions governing the formation of cellulose

Pellicles which gave reactions for cellulose were formed by *A. acetigenum* E.A. from soluble starch, dextrin, sucrose, maltose, lactose, glucose, fructose, galactose, α-methyl-D-glucoside, β-methyl-D-glucoside, salicin, arabinose, xylose, rhamnose, mannitol, erythritol, glycerol, and ethylene glycol, respectively. Tarr & Hibbert (1931) reported the inability of *A. xylinum* to form cellulose from the α- and β-methyl-D-glucosides, three pentoses, erythritol and ethylene glycol, respectively. They did not ascertain whether their organism could utilize salicin.

A. acetigenum E.A., *A. acetigenum* (Henneberg) Bergey *et al.*, National Collection of Type Cultures (N.C.T.C.) 5346, *A. pasteurianum* (an atypical strain) N.C.T.C. 613 and *A. kützingianum* (Hansen) Bergey *et al.*, N.C.T.C. 3924, produced cellulose in a medium (Henneberg, 1926) consisting of $(NH_4)_2SO_4$, 3 g.; KH_2PO_4, 3 g.; $MgSO_4.7H_2O$, 2 g.; glucose, 20 g. and ethanol (added after sterilization) 25 g., together with water to 1 l. Equally vigorous or more profuse growth could be obtained by substituting corn-steep liquor (C.S.L.), 1–2% (v/v), or Witte's peptone 0·2% (w/v) in place of or in addition to the ethanol. The same adjuncts also stimulated growth in yeast-water (made by boiling 75 g. of brewery pressed yeast for 30 min. in 1 l. of water) to which glucose or another suitable carbohydrate had been added. In some cases addition of sterile $CaCO_3$ (precipitated chalk) improved the yield of cellulose. In media containing

ethylene glycol, however, the addition of $CaCO_3$ prevented cellulose formation.

The cultures were developed in the flat, circular, glass dishes formerly employed in penicillin production (Clayton, Hunwicke, Hanes, Robinson & Andrews, 1944); these were charged with medium to a depth of 1 cm. and, after inoculation, were incubated at 30°.

When *A. acetigenum* E.A. was grown on glucose in yeast-water the yield of cellulose was improved by addition both of $CaCO_3$ and C.S.L. (Table 1). The yields from some substances other than glucose are given in Table 2. Yeast-water was employed in two only of the cases shown in Table 2; in the other cases the volume was made up to bulk with tap water. In all cases the dishes stood without agitation during incubation.

Table 1. *Yields of cellulose formed by* Acetobacter acetigenum *E.A. from glucose*

Composition of medium (to 200 ml. with yeast-water)	Time of incubation at 30° (days)	Purified cellulose: Yield (g.)	Purified cellulose: As % of wt. of glucose initially present
Glucose, 5 g.	10	0·11	2·2
Glucose, 5 g.; $CaCO_3$, 2 g.	10	0·42	8·4
Glucose, 10 g.	12	0·18	1·8
Glucose, 10 g.; $CaCO_3$, 2 g.	12	0·50	5·0
Glucose,,10 g.; $CaCO_3$, 2 g.; C.S.L. 4 ml.	10	0·71	7·1
Glucose, 10 g.; C.S.L., 4 ml.	15	0·64	6·4
Glucose, 36 g.; $CaCO_3$, 2 g.	10	1·08	3·0

Chemical examination of the bacterial cellulose

Both strains of *A. acetigenum* gave higher yields of pellicle material than were obtained from corresponding cultures of *A. pasteurianum* and *A. kützingianum*. All the pellicles formed from the different substrates by *A. acetigenum* E.A. and those formed from glucose by *A. acetigenum* N.C.T.C. 5346, by *A. pasteurianum* N.C.T.C. 613 and by *A. kützingianum* N.C.T.C. 3924, after purification, gave the following reactions for cellulose: they were soluble in H_2SO_4 (72%, w/w) and in cuprammonium sulphate, being precipitated in flocculent condition on addition of excess of acidulated water. They were soluble also in a solution of $ZnCl_2$ in HCl and they could be bleached like cotton and dyed with direct cotton dyes such as congo red, benzopurpurin and sky-blue F.F.S. They could be hydrolysed by H_2SO_4 with production of a substance giving reactions of glucose.

Membranes from cultures of *A. acetigenum* E.A., *A. pasteurianum* and *A. kützingianum* on glucose media were purified by thorough washing with distilled water, treatment for 10 hr. with cold aqueous NaOH solution (2%, w/v), followed by further washings with water, dilute acetic acid and, finally, with distilled water for 12 hr. They were then dried in air and finally in a vacuum desiccator.

(Material formed by *A. acetigenum* E.A.: Found: C, 44·7; H, 6·7; by *A. pasteurianum*: Found: C, 45·3; H, 6·8; by *A. kützingianum*: Found: C, 43·8; H, 6·4; calc. for $(C_6H_{10}O_5)_n$: C, 44·5; H, 6·2%.)

Pellicle material from cultures of *A. acetigenum* E.A. on glucose and glycerol media, respectively, was purified in the manner stated above and was treated as follows:

(*a*) *Conversion to glucose.* Membrane (0·5 g.) from a glucose culture was dissolved in H_2SO_4 (72%, w/w) and the solution was diluted with fifty times its volume of water and boiled for 5 hr. with periodical addition of sufficient water to maintain the original volume. After neutralization with Na_2CO_3, samples reduced Fehling's solution, Barfoed's reagent and ammoniacal $AgNO_3$ solution. The bulk of the boiled liquid was filtered and divided into two portions, one of which, on appropriate treatment with phenylhydrazine acetate, yielded phenylglucosazone, m.p. 209° (decomp.), identical in crystalline form with an authentic specimen prepared from pure glucose. The other portion was warmed for several hours with 2:4-dinitrophenylhydrazine in 2N-HCl and the orange-red, solid product after recrystallization had m.p. 264° (decomp.) and was indistinguishable from the 2:4-dinitrophenylosazone of glucose (m.p. 264°, decomp.) prepared from British Drug Houses Ltd. glucose (A.R.). (Found: N, 20·6. Calc. for $C_{18}H_{18}O_{12}N_8$: N, 20·8%.)

The membrane from a culture on a glycerol medium was purified and dried in a vacuum desiccator. (Found: C, 44·2; H, 6·6. Calc. for $(C_6H_{10}O_5)_n$: C, 44·5; H, 6·2%.) A portion

Table 2. *Yields of cellulose formed by* Acetobacter acetigenum *E.A. from substances other than glucose*

	Composition of medium (to 200 ml.)	Days of incubation at 30°	Purified cellulose: Yield (g.)	Purified cellulose: As % of wt. of carbon source initially present
1	Mannitol, 10 g.; Henneberg salts; C.S.L., 4 ml.	28	0·47	4·7
2	Glycerol, 10 g.; Henneberg salts; C.S.L., 4 ml.	28	0·35	3·5
3	Glycerol, 20 g.; Henneberg salts; C.S.L., 4 ml.	35	1·00	5·0
4	Ethylene glycol, 10 g.; Henneberg salts; C.S.L., 4 ml.	28	0·10	1·0
5	Ethylene glycol, 15 g.; C.S.L., 4 ml.; yeast-water	35	0·90	6·0
6	Arabinose, 5 g.; Henneberg salts (with the weight of $(NH_4)_2SO_4$ reduced to 1 g./l.); peptone, 0·4 g.	23	0·12	2·3
7	Xylose, 5 g. Other constituents as for arabinose	23	0·11	2·2
8	Rhamnose, 5 g. Other constituents as for arabinose	23	0·10	2·0
9	Erythritol, 5 g.; C.S.L., 4 ml.; yeast-water	23	0·10	2·0
10	Soluble starch, 3 g.; K_2HPO_4, 0·6 g.; peptone, 0·4 g.	34	0·3	10·0

of it was hydrolysed with H_2SO_4, yielding a solution from which the phenylosazone (m.p. 209°) and the 2:4-dinitrophenylosazone of glucose (m.p. 264°) were prepared.

(*b*) *Conversion to a mixture of octa-acetyl cellobiose and α-penta-acetyl glucose.* Membrane (1 g.) from a culture on a glucose medium was treated with acetic anhydride (8 ml.) and conc. H_2SO_4 (0·2 ml.) during 16 days at 45°. The product was isolated by the procedure of Barsha & Hibbert (1934) and purified. Yield, 0·7 g. of m.p. 226°. The mixed melting point with an authentic sample of octa-acetyl cellobiose (m.p. 226°), prepared from purified cotton wool, was identical. In a further experiment 2 g. of membrane were acetolysed with acetic anhydride (16 ml.), glacial acetic acid (8 ml.) and conc. H_2SO_4 (2 ml.) at room temperature for 24 days. The crude product (dry wt. 1·2 g.) yielded 0·3 g. of pure octa-acetyl cellobiose (m.p. 226°) and 0·2 g. of α-penta-acetyl glucose of m.p. 110°. The mixed melting point of the latter with an authentic sample of α-penta-acetyl glucose kindly supplied by Prof. F. Challenger was identical.

(*c*) *Conversion to cellulose triacetate.* The material (1·1 g.) obtained from a glucose medium was boiled gently for 2 hr. with acetic anhydride (20 ml.), glacial acetic acid (10 ml.) and fused $ZnCl_2$ (1 g.). On pouring the mixture into water the product separated, and was purified by addition of methanol to its solution in acetone, giving 1·6 g. (96% yield) of cellulose triacetate which was indistinguishable from the product prepared by the same method from 1·2 g. of purified cotton wool. Portions of each product were subjected to further purification by precipitation from solution in $CHCl_3$ by addition of methanol. (Acetate from bacterial cellulose: Found: C, 49·1; H, 5·8; $CH_3.CO$–, 44·7; acetate from cotton wool: Found: C, 49·2; H, 5·5; $CH_3.CO$–, 45·5; Calc. for $C_6H_7O_5(-CO.CH_3)_3$: C, 50·0; H, 5·6; $CH_3.CO$–, 44·8%.)

(*d*) *Acetolysis at low temperature with formation of β-penta-acetyl glucose.* Acetic anhydride (8 g.), glacial acetic acid (4 g.) and conc. H_2SO_4 (1 ml.) were mixed at −10° and 1 g. of membrane from a culture on a glycerol medium was added in small portions, the temperature not being allowed to exceed 0°. After several hours at 0° the material had dissolved and 16 days later the solution was poured into water (400 ml.). The weight of the white flocculent precipitate after washing and drying was 0·5 g. It was dissolved in $CHCl_3$, 3 vol. of methanol were added, and the whole was filtered and concentrated, yielding finally a small quantity of crystalline material. This was redissolved in $CHCl_3$, boiled with charcoal and allowed to stand at room temperature, when crystals of m.p. 129–133° were obtained. The mixed melting point with an authentic sample of β-penta-acetyl glucose, kindly supplied by Prof. F. Challenger, was identical.

Detection of intermediates during the formation of cellulose

Identification of dihydroxyacetone (or glyceraldehyde) in cultures on a glycerol medium. In a medium consisting of glycerol (10%, w/v) in Henneberg's solution of salts together with C.S.L. (2%, v/v) *A. acetigenum* E.A. formed a substance which reduced Fehling's solution in the cold. The reducing value of the culture liquid, estimated by the Willstätter-Schudel method, showed a considerable rise on the 35th day. At this time accumulation of cellulose had ceased. In another experiment, material in solution which could be precipitated by 2:4-dinitrophenylhydrazine was so removed periodically, and at each of these times the weight of cellulose was ascertained (Table 3). All the precipitates after crystallization from pyridine were obtained as small red prisms of m.p. 284°. The mixed melting point with a sample of the 2:4-dinitrophenylosazone of dihydroxyacetone (or of glyceraldehyde), prepared from Merck's dihydroxyacetone, was identical. (Found: N, 25·3. Calc. for $C_{15}H_{12}O_8N_8$: N, 25·0%.) From similar glycerol cultures the triose was obtained as the phenylosazone, which crystallized from benzene in golden-yellow needles of m.p. 132°. The mixed melting point with an authentic specimen (m.p. 132°) prepared from Merck's dihydroxyacetone was identical. (Found: C, 66·8; H, 6·2; N, 21·0. Calc. for $C_{15}H_{16}ON_4$: C, 67·2; H, 6·0; N, 20·9%.)

Table 3. *Production of cellulose and of dihydroxyacetone by* Acetobacter acetigenum *E.A. in* 200 *ml. of a medium containing glycerol* (10%, *w/v*)

Time of incubation at 30° (days)	Purified cellulose: Yield (g.)	Purified cellulose: As % of wt. of glycerol initially present	Wt. of 2:4-dinitrophenylosazone of dihydroxyacetone (g.)
17	0·70	3·5	1·68
20	0·82	4·1	1·72
28	0·83	4·2	1·78
35	0·97	4·9	1·84
49	0·90	4·5	3·60

Identification of glycolaldehyde in cultures on an ethylene glycol medium. Cultures were developed in media containing (*a*) ethylene glycol, 21 g.; $CaCO_3$, 9 g.; C.S.L., 4 ml.; yeast-water to 600 ml.; (*b*) ethylene glycol, 21 g.; $CaCO_3$, 9 g.; yeast-water to 600 ml.; and (*c*) ethylene glycol, 14 g.; C.S.L., 4 ml. yeast-water to 400 ml.

After 15 days at 30° a thick pellicle had developed on (*c*), but (*a*) and (*b*) were without surface growth. The pellicle from (*c*) after washing and drying weighed 0·92 g., equivalent to 6·6% on the glycol initially in solution. Each medium was filtered and in all cases samples on treatment with HCl and naphthoresorcinol gave the colour reaction for glyoxylic acid (Neuberg, 1910). Each filtrate was then mixed with twice its volume of a solution of 2:4-dinitrophenylhydrazine in 2N-HCl; the resultant precipitates weighed (*a*) 0·2042 g., (*b*) 0·0520 g. and (*c*) 0·2878 g., respectively. Precipitates (*a*) and (*c*) were recrystallized first from pyridine, then from nitrobenzene, after which each had m.p. 326° (decomp.). In each case the mixed melting point with an authentic sample of the 2:4-dinitrophenylosazone of glycolaldehyde (m.p. 326–329°, decomp.) prepared from glycolaldehyde obtained by the method of Fenton & Jackson (1899) was 326° (decomp.). (Found: N, 26·4, 26·6. Calc. for $C_{14}H_{10}O_8N_8$: N, 26·8%.)

In another experiment glycolaldehyde was isolated from an ethylene glycol medium as the phenylosazone which, after crystallization from absolute ethanol, had m.p. 170° (decomp.). The mixed melting point with a sample prepared from authentic glycolaldehyde (Fenton & Jackson, 1899) was identical. (Found: C, 70·4; H, 5·9. Calc. for $C_{14}H_{14}N_4$: C, 70·6; H, 5·8%.)

DISCUSSION

The formation of cellulose from ethylene glycol by way of glycolaldehyde, as now described, is of interest principally from the fact that, so far as the authors are aware, it constitutes the first instance in which the transformation of this aldehyde to a higher carbohydrate has been established experimentally by use of living cells. The only previously recorded observation of the conversion of ethylene glycol to glycolaldehyde by a micro-organism appears to be that of Goepfert & Nord (1942) who isolated glycolaldehyde, in the form of its dimedone derivative, from a culture of a *Fusarium* species on a glycol medium. The bacterial conversion of glycerol, via dihydroxyacetone (or glyceraldehyde) to cellulose, is likewise of interest, since glyceraldehyde has not hitherto been identified as a metabolite in reactions leading to cellulose.

SUMMARY

1. A strain of *Acetobacter acetigenum*, isolated from East African vinegar, formed cellulose from all of eighteen carbohydrates, or alcohols related to carbohydrates, which were submitted to its action.

2. By its ability to convert to cellulose the α- and β-methyl-D-glucosides, three pentoses, erythritol and ethylene glycol, respectively, the new organism showed itself to be enzymically more active than *Acetobacter xylinum* which, in previous studies of cellulose formation (Tarr & Hibbert, 1931), proved unable to utilize these substances for this purpose.

3. During the formation of cellulose from ethylene glycol and from glycerol there were formed glycolaldehyde and glyceraldehyde, respectively, and these were characterized as their osazones.

4. It was shown that *A. acetigenum* Henneberg, *A. pasteurianum* (an atypical strain) and *A. kützingianum* Hansen can each synthesize cellulose when grown on suitable media.

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Studies in Detoxication

37. METABOLISM OF BENZENE. EXAMINATION OF THE GLUCURONIDE FRACTION OF RABBIT URINE AFTER ADMINISTRATION OF BENZENE. ISOLATION OF PHENYLGLUCURONIDE

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In 1881 Schmiedeberg obtained a gum from the urine of dogs fed with benzene and concluded that because the gum gave phenol and tests for glucuronic acid, it contained phenylglucuronide, a compound which had not then been described and which was isolated for the first time by Külz in 1890 from the urine of rabbits fed with phenol.

No glucuronide has, hitherto, been isolated from urine after feeding benzene. The urine passed after feeding benzene will be referred to in this paper as benzene urine. Porteous & Williams (1949*b*) examined the ethereal sulphate fraction of benzene urine and found it to contain phenol, catechol, quinol and hydroxyquinol. We have now examined the

glucuronide fraction and find it to contain mainly phenol with only small amounts of polyphenols. Phenylglucuronide was isolated from this fraction as the crystalline amide and triacetyl methyl ester.

EXPERIMENTAL

Preparation of reference compounds

Phenylglucuronide methyl ester. Phenylglucuronide (m.p. 160°) was prepared by feeding rabbits with phenol (yield 6·1 g. from 5 g. phenol) according to Porteous & Williams (1949*a*). The glucuronide (4 g.) dissolved in methanol (20 ml.) was methylated with ethereal diazomethane. Evaporation of the solvent gave *β-phenyl-D-glucuronide methyl ester* (3·9 g.) as a pale-yellow gum which was crystallized with great difficulty from ether-methanol. It formed colourless prisms m.p. 153° and $[\alpha]_D^{23°} - 63° \pm 1°$ (*c*, 1 in methanol). (Found: OMe, 10·6. $C_{13}H_{16}O_7$ requires OMe, 10·9 %.) The compound readily decomposes on heating to about 80°, forming a dark acidic gum.

Triacetyl β-phenyl-D-glucuronide methyl ester. The above ester (2 g.) in 10 ml. pyridine was treated with acetic anhydride (8 ml.) at 5°. After keeping the mixture for an hour, it was poured into an excess of water whereby a pale-yellow viscous gum separated which crystallized on keeping at 0°. The crystals were dissolved in $CHCl_3$ (25 ml.) and the solution was washed several times with 2N-HCl and finally with water. After drying the solution over anhydrous Na_2SO_4 and then evaporating, the *triacetyl methyl ester* (2·8 g.) was obtained as a crystalline solid. Recrystallization from aqueous ethanol gave the compound as colourless needles (2·1 g.) m.p. 116° and $[\alpha]_D^{22°} - 33° \pm 0·5°$ (*c*, 1 in $CHCl_3$), $-32·5°$ (*c*, 1 in ethanol). (Found: OMe, 7·5. $C_{19}H_{22}O_{10}$ requires OMe, 7·6 %.)

Phenylglucuronidamide. The above triacetyl methyl ester (1 g.) was dissolved in methanol (10 ml.) and the solution saturated with dry NH_3. After keeping for an hour, the solution was diluted with 50 ml. water and on standing a crystalline solid (340 mg.) separated. The *β-phenyl-D-glucuronidamide* was recrystallized from aqueous acetone giving colourless sheaves of needles, m.p. 256° (decomp.) and $[\alpha]_D^{22°} - 70° \pm 5°$ (*c*, 0·1 in methanol). (Found: C, 53·8; H, 5·6; N, 5·0. $C_{12}H_{15}O_6N$ requires C, 53·5; H, 5·6; N, 5·2 %.) The amide was only sparingly soluble in hot water, methanol and ethanol, but readily soluble in hot aqueous acetone.

Triacetyl phenylglucuronidamide. The above amide (100 mg.) was suspended in pyridine (5 ml.) and treated with acetic anhydride (3 ml.) at 5°. The amide dissolved in 2 hr. at room temperature and then the mixture was poured into water (100 ml.). No crystals separated. The solution was extracted with $CHCl_3$ and the extract washed with 2N-HCl and water, and then dried with anhydrous Na_2SO_4. Evaporation of the $CHCl_3$ left a crystalline solid which on recrystallization from 10 % aqueous ethanol gave the *triacetyl amide* as colourless needles, m.p 158° and $[\alpha]_D^{22°} - 33° \pm 1°$ (*c*, 1 in $CHCl_3$). (Found: C, 54·5; H, 5·3. $C_{18}H_{21}O_9N$ requires C, 54·7; H, 5·4 %.) The compound was readily soluble in ethanol but not in water.

Isolation of phenylglucuronide derivatives from benzene urine

The glucuronide gum. Five rabbits were fed a total of 50 g. of benzene in 6 days and the basic lead acetate precipitate of the urine (1·4 l.) was prepared in the usual manner. After removal of the Pb with H_2S and concentration of the resulting solution, the glucuronide gum was obtained. The gum was dissolved in the minimum of water and the solution poured into ethanol (400 ml.). The precipitate formed was filtered off and the filtrate evaporated to dryness *in vacuo* at 30–35°. The residue was again dissolved in the minimum of water and poured into ethanol. In this way, the glucuronide gum was separated into an ethanol-soluble fraction (*A*) and an ethanol-insoluble fraction (*B*). The latter appeared to be an inorganic salt of the glucuronide. *B* was therefore dissolved in 20 ml. N-H_2SO_4 and poured into excess ethanol. The precipitate of inorganic sulphates was removed by filtration and the ethanolic solution of the glucuronide adjusted to pH 4 with Na_2CO_3 solution and again filtered. The filtrate was then evaporated *in vacuo* at 30–35° to a gum. The yield of purified glucuronide gums was *A*, 7·2 g. and *B*, 4·8 g. *A* contained no ethereal sulphate, but *B* contained small amounts.

Isolation of triacetylphenylglucuronide methyl ester. The above gums *A* and *B* were dissolved separately in the minimum quantity of methanol and methylated with an ethereal solution of diazomethane. After keeping overnight, the solution was filtered and the insoluble material dissolved in methanol and again methylated. The insoluble material left after the second methylation gave only a weak naphthoresorcinol reaction and was therefore discarded. Evaporation of the solvents gave the methylated glucuronide as pale-yellow gums, *A* yielding 6 g. and *B* 3·8 g. The two methylated gums were now separately acetylated in pyridine with 10 equiv. of acetic anhydride. After keeping overnight at 0°, the mixtures were poured into water. The semi-crystalline material which formed was filtered off, dissolved in $CHCl_3$ and washed with 2N-HCl followed by water. After drying over anhydrous Na_2SO_4, the $CHCl_3$ was evaporated and the residue recrystallized from aqueous ethanol. Gum *A* yielded 0·85 g. of triacetylphenylglucuronide methyl ester, m.p. and mixed m.p. 116° and $[\alpha]_D^{22°} - 33°$ (*c*, 1 in ethanol) and gum *B* 1·1 g., m.p. 116°.

The residues from the isolation of the crystalline triacetyl methyl ester from both *A* and *B* were dissolved in methanol (20 ml.) and the solution saturated with ammonia at 0°. Gum *A* yielded 40 mg. of phenylglucuronidamide, m.p. and mixed m.p 256°, and gum *B* 135 mg. On acetylation both these amides yielded triacetylphenylglucuronidamide, m.p. and mixed m.p. 158°.

The total yield of crystalline phenylglucuronide derivatives isolated from the glucuronide fraction of benzene urine corresponded to 1 % of the dose. No other glucuronide was found.

Examination of the glucuronide fraction for dihydrodihydroxybenzene. Direct ether extraction of benzene urine at pH 7 failed to yield any material which spectroscopically might be a benzene-diol. The glucuronide fraction from the urine of rabbits which had collectively received 54 g. of benzene was therefore prepared. It was purified by dissolution in water and pouring into ethanol as above. Insoluble inorganic material which was precipitated was discarded. The purified gum after drying *in vacuo* at room temperature had $[\alpha]_D^{22°} - 48°$ (*c*, 1 in ethanol). Spectroscopic examination indicated that it was mainly phenylglucuronide and there was no pronounced absorption in the 260 mμ. region which might be indicative of the presence of a hexa-1:3-diene derivative.

Hydrolysis of the 'benzene' glucuronide with β-glucuronidase. A preparation of β-glucuronidase from ox spleen, purified by

precipitation between the limits of 1·25 and 1·75 M-$(NH_4)_2SO_4$, was prepared. The activity of the preparation was determined according to Spencer & Williams (1951). The 'benzene' glucuronide gum (2 g.) was dissolved in 400 ml. of 0·2 M-acetate buffer of pH 5·2 and treated with 60 ml. of β-glucuronidase solution containing 22,500 *p*-chlorophenyl units (1 *p*-chlorophenyl unit of β-glucuronidase activity is defined as that amount of the enzyme which liberates 1 μg. of *p*-chlorophenol from $1·5 \times 10^{-2}$ M solution of *p*-chlorophenylglucuronide in 0·2 M-acetate buffer at pH 5·2 and 37° in 1 hr.). After incubation for 24 hr. a further 15,000 units (40 ml.) of enzyme extract was added and incubation continued for a further 3 days. The mixture was now adjusted to pH 7 with N-NaOH and continuously extracted with ether for 18 hr. Evaporation of the ether gave a dark residue consisting mainly of phenol. The phenol was removed by dissolving the residue in water (25 ml.) and evaporating. The phenol-free residue was shown by paper chromatography to contain catechol, quinol and hydroxyquinol. The ultraviolet absorption spectrum of the residue was the same before and after heating with N-HCl for 30 min. at 95°, and no phenol was produced by the acid treatment. Thus no evidence was obtained that a benzene-dihydrodiol occurred in the 'benzene' glucuronide.

Examination of the 'benzene' glucuronide by paper chromatography. The glucuronide gum from benzene urine often contained traces of ethereal sulphates which might give rise to the small amounts of catechol, quinol and hydroxyquinol found in the glucuronide gum. Accordingly, 2 g. of the glucuronide gum were heated with 25 ml. N-HCl at 95° for 30 min. to hydrolyse any ethereal sulphates present and then exhaustively extracted with ether. The extract was freed of phenol by evaporation and the residue chromatographed on paper using benzene (40), acetic acid (50) and water (10) as solvents (cf. Bray, Thorpe, & White 1950; for other solvents for separating phenols, see Bate-Smith, 1949; Evans, Parr & Evans, 1949). The chromatogram, on treatment with a solution of diazotized *p*-nitraniline and alkali according to Bray *et al.* (1950), showed the presence of catechol, quinol and hydroxyquinol, but not of resorcinol, pyrogallol or phloroglucinol. R_F values under our conditions and the colours given with diazotized *p*-nitraniline and alkali are quoted in Table 1. The residual glucuronide solution was now made 5 N with respect to HCl and heated for 1 hr. at 95° to hydrolyse glucuronides. The extraction with ether, separation of phenol and paper chromatography was repeated as above, and again catechol, quinol and hydroxyquinol were detected. By colour reactions in solution and on paper chromatograms it was concluded that the benzene glucuronide contained roughly 1 % of each of catechol and quinol glucuronides and about 0·2 % of hydroxyquinol.

Table 1. *R_F values and colour reactions of the di- and tri-hydroxybenzenes*

(Solvent benzene (40), acetic acid (50) and water (10); chromatograms run for 15 hr. on Whatman no. 1 paper.)

Compound	R_F value	Colour given with diazotized *p*-nitraniline and Na_2CO_3
Catechol	0·68	Violet-green
Resorcinol	0·67	Orange
Quinol	0·66	Grey-green
Pyrogallol	0·60	Brown-pink
Hydroxyquinol	0·56	Violet
Phloroglucinol	0·50	Orange-brown

DISCUSSION

Porteous & Williams (1949*a*, *b*) examined the ethereal sulphate fraction of benzene urine and proved that it contained phenol, and the polyphenols catechol, quinol and hydroxyquinol. Although phenol is a major component of this fraction, the polyphenols are also present in considerable amounts and probably constitute about a half of the total phenols of the ethereal sulphate fraction. Our present examination of the glucuronide fraction of benzene urine shows that it is almost entirely made up of phenylglucuronide, the polyphenols occurring only in very small amounts. There is therefore a considerable quantitative difference in the nature of the phenols of the ethereal sulphate and of the glucuronide fractions of benzene urine. At the moment there seems to be no clear explanation of this difference, unless we conclude that phenylsulphuric acid under some conditions is readily oxidized to polyphenols whereas phenylglucuronide is not. Garton & Williams (1949), however, found that neither of these compounds on injection into rabbits was appreciably oxidized to polyphenols. However, it does appear that the formation of polyphenols *in vivo* is in some way connected with sulphur compounds.

Porteous & Williams (1949*a*) suggested that dihydrodihydroxybenzene might be a precursor of some of the phenols of benzene urine. Although a similar compound has been isolated from chlorobenzene urine (Smith, Spencer & Williams, 1950), we have been unable to detect it in benzene urine. Attempts to synthesize 1:2-dihydro-1:2-dihydroxybenzene in this laboratory have been unsuccessful.

SUMMARY

1. The glucuronide fraction from the urine of rabbits dosed with benzene has been examined and shown to be almost entirely phenylglucuronide which was isolated as its crystalline triacetyl methyl ester.

2. Detailed examination of the glucuronide fraction using β-glucuronidase and paper chromatography showed that this fraction contained only very small amounts of the polyphenols, catechol, quinol and hydroxyquinol. The dihydrodiol of benzene was not detected.

3. The methyl ester, triacetyl methyl ester, amide and triacetyl amide have been prepared from phenylglucuronide.

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38. THE METABOLISM OF BENZENE. (*a*) THE DETERMINATION OF PHENYLMERCAPTURIC ACID IN URINE. (*b*) MERCAPTURIC ACID EXCRETION BY RABBITS RECEIVING BENZENE

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We have shown that some 60 % of a dose of benzene administered to rabbits can be accounted for by oxidation to phenols (Porteous & Williams, 1949*a*, *b*) and by elimination in the unchanged state (Parke & Williams, 1950). Benzene, however, is also excreted as phenylmercapturic acid and Zbarsky & Young (1943) have proved this by the isolation of the mercapturic acid in a maximum yield of 0·37 % of the dose from the urine of rats dosed with benzene. Although Witter (1945) failed to isolate phenylmercapturic acid from the urine of rabbits receiving benzene, Drummond & Finar (1937) have suggested that these animals also excrete this conjugate. In the present paper we shall show that rabbits do, in fact, excrete phenylmercapturic acid and that, determined by the quantitative methods developed here, the amount excreted is about 1 % of the dose.

EXPERIMENTAL

*Preparation of phenyl-*L*-cysteine derivatives.* Phenyl-L-cysteine has been synthesized by Zbarsky & Young (1943), but we were unable to reproduce their conditions exactly. The present method has produced rather better yields. L-Cystine (5 g.) was reduced by boiling for 2 hr. in 100 ml. N-H_2SO_4 with 3 g. of Zn dust, a few pieces (total, 1 g.) of granulated Zn (A.R.) being added periodically. The solution was filtered hot and, in order to facilitate the formation of the cuprous mercaptide, 1 ml. of conc. HCl was added (cf. Pirie, 1931). The hot solution was now treated with freshly precipitated Cu_2O (3·5 g. in 20 ml. water) which slowly dissolved on shaking to give a green solution. When the addition of the oxide is near completion the mercaptide may tend to come out of solution as a white precipitate, but this can readily be redissolved by adding a little 2N-H_2SO_4. Excess Cu_2O should be avoided, since it tends to co-precipitate with the mercaptide as a dark-brown spongy solid. To the solution of cuprous mercaptide cooled to 0°, there was added a solution of diazotized aniline (8 g.) in 2N-H_2SO_4 (120 ml.). A brisk evolution of N_2 occurred with the formation of a buff precipitate. After 30 min. at 0°, the solution was heated at 60–70° for 30 min., filtered and extracted with ether (50 ml.) to remove phenol. The resulting green solution was saturated with H_2S to remove Cu and, after filtration of the copper sulphide, the solution was boiled *in vacuo* to remove excess H_2S. The solution was then neutralized with conc. NH_3 solution followed by glacial acetic acid (5 ml.) and after keeping at 0° overnight, the precipitated phenyl-L-cysteine was collected by filtration. At this stage, the compound was heavily contaminated with cystine which was largely removed by repeated dissolution in N-NaOH (charcoal) and precipitation with 2N-acetic acid. The material was finally recrystallized from 50 % aqueous ethanol in which cystine is very sparingly soluble (Clarke & Inouye, 1931). The product (2·2 g.) was obtained as white glistening plates, m.p. 180–181°; $[\alpha]_D^{23°}$ +10° (*c*, 1 in 0·1 N-NaOH). (Zbarsky & Young (1943) give m.p. 170–172° and $[\alpha]_D^{25°}$ +11° in 0·1 N-NaOH.) A sample prepared by debromination and deacetylation of biosynthetic *p*-bromophenylmercapturic acid (Zbarsky & Young, 1943) had m.p. 180–181° and $[\alpha]_D^{23°}$ +10° (*c*, 1 in 0·1 N-NaOH).

p-Bromophenyl-L-cysteine was similarly prepared from cystine (5 g.) and diazotized *p*-bromoaniline (7·5 g.) (yield, 2·6 g.). It formed colourless, glistening plates, m.p. 193° (not depressed by a sample, m.p. 194°, prepared by deacetylation of biosynthetic *p*-bromophenylmercapturic acid according to Baumann & Preusse, 1881), and $[\alpha]_D^{23°}$ +20° (*c*, 1 in 0·1 N-NaOH).

*p-Chlorophenyl-*L*-cysteine.* This compound has not been previously described, although the DL-form (m.p. 202°) has been synthesized by Behringer & Fackler (1949). It was prepared from cystine (5 g.) and diazotized *p*-chloraniline (5·5 g.) as described above for phenylcysteine. The yield was 2 g. after recrystallization from 50 % ethanol. It formed colourless glistening elongated plates, m.p. 190°, $[\alpha]_D^{23°}$ +18°

(*c*, 1 in 0·1 N-NaOH). (Found: C, 46·9; H, 4·7; Cl, 15·1. $C_9H_{10}O_2NSCl$ requires C, 46·7; H, 4·4; Cl, 15·3 %.) A sample was also prepared by heating biosynthetic *p*-chlorophenylmercapturic acid (0·1 g.) under reflux with 5 ml. 4 N-H_2SO_4 for 45 min. After cooling, the solution was neutralized with NH_3 solution, and after keeping overnight the precipitate of *p-chlorophenyl-L-cysteine* was washed with water and recrystallized from 50 % aqueous ethanol (yield, 50 mg.) (m.p. 189–190°; $[\alpha]_D^{23°}$ +19° (*c*, 1 in 0·1 N-NaOH)). (Found: Cl, 14·9 %.)

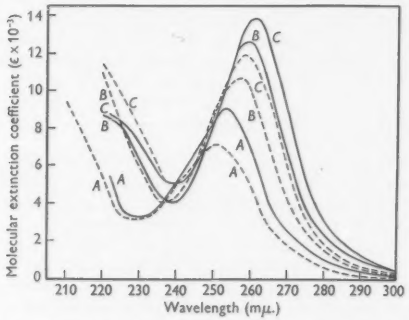

Fig. 1. Ultraviolet absorption spectra of phenyl-L-cysteine (*A*), *p*-chlorophenyl-L-cysteine (*B*) and *p*-bromophenyl-L-cysteine (*C*) in 0·1 N-NaOH (———) and 0·1 N-HCl (– – – –). See also Table 1.

L-Phenylmercapturic acid (m.p. 142°; $[\alpha]_D^{22°}$ –21° in ethanol) was prepared by acetylation of phenyl-L-cysteine according to Zbarsky & Young (1943). The yield was 0·6 g. from 1 g. of phenylcysteine. L-*p*-Chlorophenylmercapturic acid (m.p. 153°; $[\alpha]_D$ –20° in ethanol) and *p*-bromophenylmercapturic acid (m.p. 152° and $[\alpha]_D$ –13° in ethanol) were prepared biosynthetically from the urine of rabbits dosed with chlorobenzene and bromobenzene, respectively. The spectra of these compounds determined with a Unicam spectrophotometer (Model S.P. 500) are given in Figs. 1 and 2, and Table 1.

Alkaline hydrolysis of the phenylcysteines and mercapturic acids. Preliminary experiments showed that the phenylcysteines were more stable to alkaline hydrolysis than their *N*-acetyl derivatives, the mercapturic acids. These observations were investigated quantitatively as follows.

To 10 ml. portions of 0·1 % (w/v) solutions of each of phenyl-, *p*-chlorophenyl- and *p*-bromophenyl-mercapturic acids, there were added 10 ml. of 0·2, 0·3, 0·5, 0·7, 1·0 and 1·5 N-NaOH, respectively. A similar set of solutions of 0·1 % phenyl-, *p*-chlorophenyl- and *p*-bromophenyl-cysteines (made by dissolving the arylcysteine in hot water and adding a few drops of ammonia) were also set up, but the NaOH concentrations used were 2, 4, 6, 8 and 10 N. The solutions were heated on a sand bath (105°) in conical flasks capped with small beakers for 30 or 60 min. The flasks were cooled in ice, treated with 10 ml. ethanol and enough 2 N-H_2SO_4 to neutralize the alkali and provide an excess of 2 ml. After the addition of 10 drops starch solution, the thiophenols set free were titrated with 0·01 N-I_2 (1 l. N-I_2 = 1 g.mol. of mercapturic acid). The results (see Fig. 3) show that the mercapturic acids are completely hydrolysed by alkali hydroxides of less than N concentration in 0·5 hr., whereas the aryl-

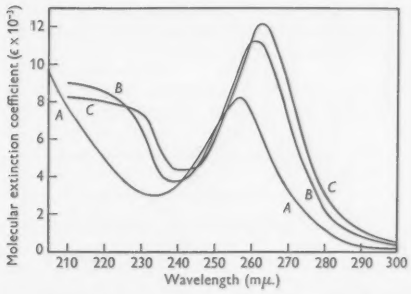

Fig. 2. Ultraviolet absorption spectra of L-phenylmercapturic acid (*A*), L-*p*-chlorophenylmercapturic acid (*B*) and L-*p*-bromophenylmercapturic acid (*C*) in ethanol. See also Table 1.

Table 1. *Optical rotations and absorption spectra of some mercapturic acids and derivatives*

Compound	Optical rotation: In 0·1 N-NaOH $[\alpha]_D^{23°}$	Optical rotation: In ethanol $[\alpha]_D^{22°}$	Absorption spectra in 0·1 N-NaOH: $\lambda_{max.}$ (mμ.)	0·1 N-NaOH: $\epsilon_{max.} \times 10^{-3}$	0·1 N-HCl: $\lambda_{max.}$ (mμ.)	0·1 N-HCl: $\epsilon_{max.} \times 10^{-3}$	Ethanol: $\lambda_{max.}$ (mμ.)	Ethanol: $\epsilon_{max.} \times 10^{-3}$
Phenyl-L-cysteine	+10°	—	253	9·1	250	7·0	—	—
p-Chlorophenyl-L-cysteine	+18°	—	260	12·6	257	10·6	—	—
p-Bromophenyl-L-cysteine	+20°	—	262	13·9	259	11·9	—	—
L-Phenylmercapturic acid	—	–21°	—	—	—	—	256–257	8·1
L-*p*-Chlorophenyl-mercapturic acid	—	–20°	—	—	—	—	260–261	11·2
L-*p*-Bromophenyl-mercapturic acid	—	–13°	—	—	—	—	262–263	12·1

cysteines require up to 5N-alkali for complete hydrolysis. The substituted phenylmercapturic acids were more readily hydrolysed than the unsubstituted derivatives.

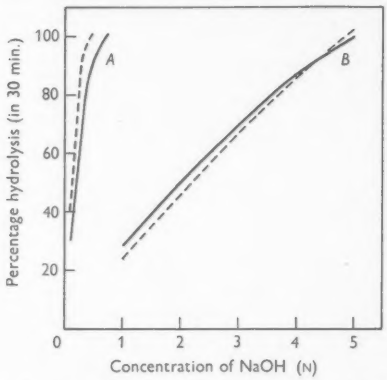

Fig. 3. Extent of hydrolysis to the thiophenols of phenylmercapturic acids (*A*; unsubstituted, ———; *p*-chloro- and *p*-bromo derivatives, – – – –) and phenylcysteines (*B*; unsubstituted, ———; *p*-chloro and *p*-bromo derivatives, – – – –) by various concentrations of NaOH in 30 min.

The determination of phenylmercapturic acid

Methods for the determination of *p*-bromophenylmercapturic acid have been devised by Stekol (1936) and Binkley (1949). In Stekol's method, the mercapturic acid was hydrolysed by 0·35N-NaOH to *p*-bromothiophenol which was then estimated iodometrically, or gravimetrically as *p*-bromophenylmercuric mercaptide. Binkley's method, which only indirectly involves the mercuric mercaptide, is colorimetric and uses Folin's reagent. Methods involving the formation of a phenylmercuric mercaptide are more specific than the iodometric method, but Stekol's gravimetric method cannot be conveniently used on a small scale. It is also clear from the preceding section that whereas 0·35N-NaOH completely hydrolyses *p*-chloro- and *p*-bromophenylmercapturic acids, more concentrated alkali is necessary for phenylmercapturic acid, whilst for the phenylcysteines 5N-NaOH may be necessary.

The effect of diet on the figures obtained by the iodometric method. Where mercapturic acids are estimated iodometrically in urine, it is necessary to determine a basal value in terms of the average iodometric titre of a day's output of normal urine before the compound to be studied can be administered. The I_2 titre for normal urine is variable unless great care is taken to maintain the animals on a constant diet. On a daily diet of 75 g. rat cubes (J. Thorley, Kings Cross, London), the average titre was 15 ml. 0·01N-I_2; on 75 g. rat cubes + 50 g. cabbage, 33 ml.; on 75 g. of an equal mixture of bran and oats, 7·5 ml.; and on 'Diet 41' (Associated London Flour Millers), 7 ml.

The iodometric method for all urines was carried out as follows. Each day's urine was made up to 200 ml. with water. To 100 ml. there was added 50 ml. 10 % (w/v) $ZnSO_4$ and 50 ml. 0·5N-NaOH, the solutions mixed and centrifuged. The clear solution (40 ml.) mixed with 5N-NaOH (10 ml.) was heated on a sand bath (105°) for 40 min. After cooling under the tap, the solution was acidified with 27 ml. of 2N-H_2SO_4. Ethanol (25 ml.) and starch solution (10 drops) were then added and the mixture titrated in an ice bath with 0·01N-I_2. A blank titration was performed on 40 ml. of the clear solution without hydrolysis with NaOH.

The turbidimetric method

The small amounts of phenylmercapturic acid expected in benzene urines rendered the iodometric method uncertain owing to the variability of the normal blanks and the presence of other iodine-consuming compounds in benzene urine (see later). Furthermore, the more specific gravimetric method was not adaptable to small amounts. However, an observation that when mercuric chloride was added to hydrolysed benzene urines stable suspensions were formed suggested a turbidimetric method.

Identity of the mercuric mercaptide. On alkaline hydrolysis phenylmercapturic acid gives thiophenol which reacts in acid ethanolic solution with $HgCl_2$ to give phenylmercuric mercaptide, $(C_6H_5S)_2Hg$. On standing, according to Lecher (1920), an equilibrium, $2C_6H_5SHgCl \rightleftarrows (C_6H_5S)_2Hg + HgCl_2$, is attained. The mercaptide, which forms colourless plates (decomposes at 152°), prepared from phenylmercapturic acid was found to be identical with that prepared from authentic thiophenol and in a gravimetric experiment, 49·1 mg. pure phenylmercapturic acid yielded 43·5 mg. of the mercaptide (calc. for $C_{12}H_{10}S_2Hg$, 43·0 mg.).

Turbidimetric estimation of phenylmercapturic acid. (*a*) *In pure solution.* The principle of the method is that phenylmercapturic acid is hydrolysed to thiophenol by alkali, then neutralized by acid. The solution is then treated with ethanol, to keep thiophenol in solution, and gelatin to stabilize the turbidity developed on adding $HgCl_2$. Turbidity is then measured in the Spekker absorptiometer.

Careful studies, which need not be detailed here, were made of the effects of the concentration of gelatin, electrolytes, $HgCl_2$ and ethanol and of the effect of pH and temperature on the stability of the turbidity produced by the formation of the phenylmercaptide. Briefly, 0·005–0·02 % gelatin gave complete stabilization, with no precipitation of the mercuric mercaptide in 1 hr. Outside this range precipitation occurred. The electrolyte mixtures, H_2SO_4–Na_2SO_4, HCl–NaCl and HOAc–NaOAc were tested for their effect; the last produced unstable suspensions, the second caused slow development of opacity, whilst the first was the most suitable at a salt concentration of about 0·5N. A concentration of 0·1 % $HgCl_2$ gave the best suspensions and below this concentration there was slow development and precipitation. A concentration of 16 % ethanol or less did not greatly affect the stability of the suspension; below 10 % the development of opacity became progressively slower and above 16 % precipitation occurred. A concentration of 10 % ethanol was adopted. The most stable suspensions were produced at a pH value of about 2. The best temperatures for development of opacity were between 10 and 20°; above 22° the mercaptide tended to dissolve and below 6° Na_2SO_4 tended to crystallize out of the solution.

Using 0·02 % gelatin, 10 % ethanol, 0·5N-Na_2SO_4, 0·1 % $HgCl_2$, pH < 2 and temperature 10–20°, opacity was found to develop rapidly and to remain stable for several hours.

The description of the preparation of the standard curve which follows gives the method finally adopted.

Reagents. Phenylmercapturic acid; N-NaOH; 2N- and 5N-H_2SO_4; 1 % (w/v) aqueous $HgCl_2$; 0·2 % (w/v) gelatin; N-Na_2SO_4 and ethanol.

Method. Phenylmercapturic acid (100 mg.) was hydrolysed by heating (sand bath; 105°) in 50 ml. of N-NaOH for 40 min. The solution was cooled and made up to 200 ml. with N-NaOH. This solution contained the equivalent of 0·5 mg./ml. phenylmercapturic acid. A fourfold dilution with N-NaOH was also prepared.

Volumes of 1–12 ml. of the latter solution were added to a series of twelve 25 ml. graduated flasks each containing 2·5 ml. ethanol, 2·5 ml. 0·2 % gelatin and from 11 to 0 ml. of N-NaOH. The solutions were mixed and cooled in ice water during the addition of 3 ml. of 5N-H_2SO_4 and 2·5 ml. of 1 % $HgCl_2$. The solutions were removed to room temperature, made up to 25 ml. with water and mixed. After 10 min. the opacities were measured in the Spekker absorptiometer using 1 cm. cells and Ilford spectrum yellow filter no. 606 and heat filter H 503. The blank employed was prepared by diluting to 25 ml. a mixture of 2·5 ml. ethanol, 2·5 ml. 0·2 % gelatin, 2·5 ml. 1 % $HgCl_2$, 12 ml. N-Na_2SO_4 and 2 ml. 2N-H_2SO_4. More concentrated solutions were set up by adding 5, 7·5, 10 and 12·5 ml. of the 0·5 mg./ml. solution of hydrolysed phenylmercapturic acid to 25 ml. flasks containing 7·5, 5, 2·5 and 0 ml. of N-NaOH and ethanol and gelatin solutions as before. The standard curve is given in Fig. 4.

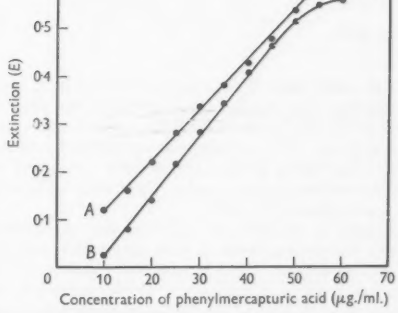

Fig. 4. Standard curves for the turbidimetric estimation of phenylmercapturic acid, *A* in pure aqueous solution, *B* in hydrolysed rabbit urine.

(*b*) *In urine.* If the above procedure is applied to hydrolysed rabbit urine diluted 1 in 5 the phenylmercuric mercaptide rapidly coagulates. Stable suspensions were readily obtained, however, if the gelatin were omitted. There is, therefore, a lyophilic substance in rabbit urine which is capable of protecting the phenyl mercuric mercaptide suspension and addition of 0·02 % gelatin acts in the same way as an excess of gelatin in pure solution and causes coagulation. The effects of pH, concentration of ethanol and $HgCl_2$ were similar to those found in pure solution.

The hydrolysed urine for the standard curve in urine was prepared by diluting a filtered 24 hr. sample of normal urine (about 100 ml.) to 200 ml. and adding 100 ml. each of 10 % $ZnSO_4$ and 0·5N-NaOH. After mixing, the solution was centrifuged and 200 ml. of the supernatant liquid hydrolysed by heating on a sand bath (105°) with 50 ml. 5N-NaOH for 40 min.

Solutions of hydrolysed phenylmercapturic acid in urine were prepared by heating phenylmercapturic acid (100 mg.) in a mixture of 40 ml. of the supernatant fraction of the urine and 10 ml. of 5N-NaOH. The solution was cooled and made up to 200 ml. with hydrolysed urine. This solution was equivalent to 0·5 mg./ml. phenylmercapturic acid. It was further diluted 1:4 for estimation.

The standard curve in urine (see Fig. 4) was then prepared in an analogous manner to that for pure solution but omitting the gelatin, by taking 1–12 ml. of the solution of hydrolysed mercapturic acid (0·125 mg./ml.) in urine, and 11–0 ml. of the hydrolysed urine.

Recoveries of phenylmercapturic acid from urine were studied as follows. Rabbit urines (24 hr. samples) were diluted to 200 ml. and various volumes (up to 50 ml.) of a 5 mg./ml. aqueous solution of phenylmercapturic acid added, followed by 100 ml. of 10 % $ZnSO_4$ and 50 ml. N-NaOH. Each solution was diluted to 400 ml. with water, mixed and centrifuged. The supernatant solutions (160 ml.) were then heated on a sand bath with 40 ml. 5N-NaOH for 40 min. made up to 200 ml. with water and the phenylmercapturic acid estimated both turbidimetrically and iodometrically as above. If the opacities in the turbidimetric method were too great, suitable dilutions were made, prior to the formation of the phenylmercuric mercaptide, with hydrolysed urines. The I_2 titre of the normal diluted rabbit urine containing no added phenylmercapturic acid was also determined. In the turbidimetric method the normal blanks were negligible.

The recoveries of phenylmercapturic acid from urine were 94 % (iodometric) and 93 % (turbidimetric) at levels of 100–250 mg./24 hr. urine and 91 % (iodometric) and 89 % (turbidimetric) at 20 mg./24 hr. urine.

Experiments with benzene

Mercapturic acid excretion. Rabbits kept on a diet of 75 g. cubes (Associated London Flour Millers) were given benzene suspended in 50 ml. water, by means of a stomach tube made of polyvinyl plastic. Urine was collected every 24 hr. and analysed both iodometrically and turbidimetrically. For analysis each day's urine was filtered and made up to 200 ml. with water. To 100 ml. of the diluted urine there was added 50 ml. each of 10 % $ZnSO_4$ and 0·5N-NaOH. The solutions were mixed and centrifuged. The supernatant liquid (80 ml.) was then heated on a sand bath (at 105°) with 20 ml. of 5N-NaOH for 40 min. After cooling the solution was made up to 100 ml. with water and 50 ml. was used for iodometric estimation and 12·5 ml. (in duplicate) for turbidimetric estimation. (During the titrations the odour of thiophenol was always evident.) For blank estimations, 40 ml. of the unhydrolysed urine, after treatment with $ZnSO_4$, was used for the iodometric blank and 10 ml. for the turbidimetric blank. Table 2 and Fig. 5 give examples of individual results, whilst Table 3 gives a summary of all results.

The accuracy of the turbidimetric method is ±5 % at the levels of phenylmercapturic acid encountered in our experiments, but it is more specific than the iodometric method and gives no measurable blank with normal urines. The higher results given by the iodometric method appear to be due to

Table 2. *Mercapturic acid excretion by rabbits receiving benzene*

Rabbit no.	Dose of benzene (g.)	Day	Urine vol. (ml.)	Iodine titre (ml. 0·01 N-I_2)	Mercapturic acid iodometric (mg.)	Mercapturic acid iodometric % of dose	Mercapturic acid turbidimetric (mg.)	Mercapturic acid turbidimetric % of dose
19	3·18	1	75	25·2*	42	0·45	22	0·2
		2	55	30·7	54	0·6	13	0·1
		3	65	12·8	12	0·1	12	0·1
19	1·59	1	50	29·8†	56	1·2	43	0·9
		2	70	18·1	28	0·6	10	0·2

* Blank titre, 7·5 ml. 0·01 N-I_2/day.
† Blank titre, 6·0 ml. 0·01 N-I_2/day.

Table 3. *Phenylmercapturic acid excretion by rabbits receiving benzene*

Rabbit no.	Benzene fed (g.)		Phenylmercapturic acid (PA) iodometrically (mg.)	Phenylmercapturic acid (PA) iodometrically % of dose	PA excretion estimated turbidimetrically (mg.)	PA excretion estimated turbidimetrically % of dose	
16	3·20	1 g./kg.	149*	1·5	115	1·2	Average 0·81
16	3·29		118†	1·2	72	0·7	
17	3·08		76†	0·8	46	0·5	
17	3·27		77†	0·8	63	0·6	
18	3·20		126†	1·3	84	0·9	
19	3·18		108†	1·1	47	0·5	
20	3·08		124†	1·3	67	0·7	
21	3·09		141†	1·5	54	0·6	
18	1·62	0·5 g./kg.	98*	2·0	60	1·2	Average 0·98
19	1·59		84*	1·8	53	1·1	
20	1·64		70*	1·4	37	0·8	
21	1·66		97*	1·9	38	0·8	

* Two days. † Three days.

hydroxyquinol, a known metabolite of benzene (Porteous & Williams, 1949*b*). This conclusion was reached by testing the reaction of various benzene metabolites in the methods of estimation used. Aqueous ethanolic solutions of phenol, catechol, quinol, *cis-cis-* and *trans-trans*-muconic acid acidified with dilute H_2SO_4, do not react with I_2 or give a precipitate with $HgCl_2$. Hydroxyquinol, however, does react with I_2 for we found, for example, that 100 mg. hydroxyquinol in 50 ml. 10 % aqueous ethanol acidified with dilute H_2SO_4 immediately reduced 15 ml. of 0·01 N-I_2 and then absorbed further I_2 more slowly. Hydroxyquinol does not form a precipitate with $HgCl_2$.

It should be pointed out, however, that the difference between the two methods is only appreciable where the mercapturic acid is a minor metabolite and is probably insignificant where mercapturic acids are formed in large amounts as in the case of the halogenobenzenes (see Stekol, 1936; Spencer & Williams, 1950*a*, *b*).

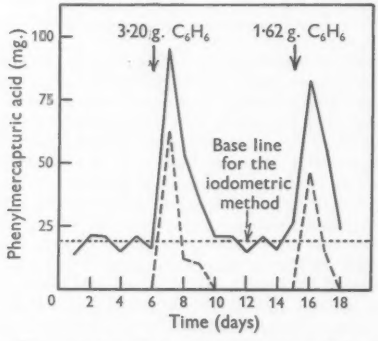

Fig. 5. The excretion of phenylmercapturic acid by rabbit no. 18 receiving benzene orally. ———, results obtained by the iodometric method; – – – –, by the turbidimetric method. The horizontal dotted line represents the average uptake of I_2 by normal rabbit urine; this uptake is not due to any phenylmercapturic acid in normal urine.

DISCUSSION

The present paper shows that phenylmercapturic acid is a metabolite of benzene in the rabbit. Estimation by the more specific turbidimetric method showed that about 0·8 % of the benzene fed at a dose level of 1 g./kg. and about 1 % at the dose level of 0·5 g./kg. is excreted as phenylmercapturic acid. Using figures obtained in an earlier paper (Parke & Williams, 1950) it can be calculated that, allowing for the benzene eliminated unchanged, 2·2 % of the benzene catabolized is excreted as a mercapturic acid at a dose of 1 g./kg. and 1·7 % at 0·5 g./kg.

We can now add one further figure to our balance sheet for the metabolism of benzene at 0·5 g./kg.

	%
Benzene eliminated unchanged	39
Benzene oxidized (and excreted as glucuronide and ethereal sulphates)	21
Benzene eliminated as phenylmercapturic acid	1

Preliminary results have shown that another metabolite of benzene, muconic acid, accounts for at least a further 1 % of the dose.

SUMMARY

1. The excretion of phenylmercapturic acid by rabbits receiving benzene has been studied.

2. The presence of phenylmercapturic acid in urine after administration of benzene has been shown by developing a specific turbidimetric method of estimation, based on the formation of phenylmercuric mercaptide from phenylmercapturic acid by alkaline hydrolysis.

3. The iodometric method for the determination of mercapturic acid has been shown to be influenced by diet and by hydroxyquinol, a metabolite of benzene, and may give high results when small amounts of mercapturic acids have to be estimated in urine.

4. By the turbidimetric method about 1 % of the benzene fed at a dose level of 0·5 g./kg. and about 0·8 % at 1 g./kg. was excreted as phenylmercapturic acid, which was found in the urine for 2–3 days after dosing.

5. Some arylcysteines and mercapturic acids have been synthesized and their ultraviolet absorption spectra recorded.

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Esterification of the Carboxyl Groups in Wool

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The carboxyl groups in wool were partially methylated with diazomethane by Rutherford, Patterson & Harris (1940) and with methyl sulphate and methyl iodide by Blackburn, Carter & Phillips (1941). In both cases titration showed that not more than 50 % of the carboxyl groups were esterified and that the reaction was non-specific and methylation of other groups occurred. Blackburn & Phillips (1944) reported that a mixture of methanol and acetic anhydride esterified the carboxyl groups, in part at least, but that some of the terminal amino groups in lysine and the peptide imino linkage were simultaneously acetylated. Fraenkel-Conrat & Olcott (1945) showed that anhydrous alcohols containing a catalytic quantity of mineral acid reacted specifically with the carboxyl groups in proteins without modifying any other group. Methanol containing 0·1 N-hydrochloric acid was used by Blackburn & Lindley (1948) to introduce 0·8 % of methyl groups into wool; this is equivalent to masking 65 % of the carboxyl groups.

Fraenkel-Conrat (1944) found that 1:2-epoxides readily esterified carboxyl groups in proteins and showed that, although epoxides also combine with amino groups, their basic nature is not suppressed and the reaction is therefore suitable for studying the effects of blocking the carboxyl group on the properties of proteins. Schlack (1947) discovered that the affinity of acidic dyes for wool was greatly increased by reacting the latter with an epoxide, and it will be shown that this results from the suppression of the ionization of the carboxyl groups.

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In this paper a quantitative study of the esterification of wool with alcohols and epoxides, the accessibility of wool to different alcohols and the stability of the ester formed are reported.

The findings of Speakman & Hirst (1933) on the mechanical properties of wool as a function of pH indicate that in the isoelectric region, approximately from pH 6 to 8, the free amino and carboxyl groups in wool exist largely in the zwitterion condition, $—NH_3^+ \ldots COO^—$. These groups, present in approximately equal numbers, mutually balance their electrical charges and probably exert an electrostatic influence on one another which decreases the swelling and increases the strength of the fibre. On combination with acid (e.g. hydrochloric acid) the configuration of this so-called salt link is changed to $—NH_3^+ \ldots Cl^- \ldots HOOC—$ and a 'Gegenion' enters the wool to bring about electrical neutrality (Alexander & Kitchener, 1950). This ion is held in the electric double layer and can take part by mass effects in ion-exchange reactions. When the carboxyl group is esterified 'Gegenions' have to be present to balance the charge of the corresponding ionized amino groups even when the fibres are in equilibrium with neutral solutions. The number of carboxyl groups esterified can therefore be determined from the reduction in the maximum acid-combining capacity of the wool since a salt link after esterification, i.e. $—NH_3^+ \ldots Cl^- \ldots ROOC—$ no longer combines with acid. It is essential that the 'Gegenion' should not be a hydroxyl ion since this, of course, would act as a base and by an ion-exchange process consume acid and give an erroneous value for the true acid-combining value of the protein. To ensure that all the 'Gegenions' were chloride ions in this investigation, the wool was repeatedly washed with solutions of potassium chloride before determining the combination with hydrochloric acid. This was particularly important in the experiments on the saponification of the esters by alkali since the first reaction was an exchange of the chloride ions by hydroxyl ions. If wool after treatment with alkali was not washed with salt solution but in distilled water only, an acid-combining value corresponding to untreated wool was obtained. However, when the esterified wool, after reaction with alkali, was equilibrated with a salt solution, then the degree of saponification could be determined from the increase in acid-combining power.

EXPERIMENTAL

Materials. For single fibre experiments Lincoln wool was used and for all other experiments knitted fabric made from high quality Merino wool.

The alcohols were dried over CaO followed by distillation from the respective diester of phthalic acid. Epichlorhydrin was obtained from Shell Chemicals Ltd., propylene oxide from Petrocarbon Ltd. and ethylene oxide from Imperial Chemical Industries Ltd. Glycidol was prepared by reaction of equivalent quantities of α-monochlorhydrin dissolved in *iso*propanol and conc. aqueous NaOH. Styrene and *iso*-eugenol epoxide were prepared by oxidation of the corresponding olefin with perbenzoic acid and peracetic acid respectively.

Esterification. When completely anhydrous conditions were required, gaseous HCl was passed into the alcohol; otherwise, conc. HCl was used. Wool dried in an air oven at 110° was treated with the alcohol at a ratio of solution to wool of 50 : 1 (v/w) in a flask fitted with reflux condenser. The epoxides were applied from 5% (w/v) aqueous solution at 95° at a solution to wool ratio of 30 : 1 (v/w).

Combination of wool with acid. The wool was equilibrated to pH 5 before measuring the acid-combining power. After esterification the wool was freed from acid by washing in running tap water for 2 hr., followed by shaking ten times for 2 min. each with distilled water and then four times with N-KCl. In this way acid is only consumed in the back titration of the carboxyl groups. After this washing the wool was placed in a solution of 0·02 N-HCl containing N-KCl at a ratio of solution to wool of 50 : 1; in this medium combination of wool with acid is at a maximum. With vigorous shaking equilibrium was attained in less than 4 min. and the amount of acid combined was determined by titration. Previous investigators who did not agitate the wool system (e.g. Speakman & Hirst, 1933) found it necessary for attainment of equilibrium to leave the wool in contact with acid for 2 days. However, Alexander, Gough & Hudson (1949*a*) showed that the rate of most reactions of ions with wool is controlled by diffusion through the liquid and is speeded up enormously by efficient agitation. When the flask containing the wool and acid was placed into an efficient shaker the time to reach equilibrium was reduced from about 2 hr. to less than 4 min. With very dilute solutions of acid more than 12 hr. were necessary to attain equilibrium without agitation, but the reaction was invariably complete after 15 min. in the shaker.

Titration curves. These were obtained by the method of Steinhardt & Harris (1940) with the modification that the solutions were placed in a shaker when equilibrium was reached in 15 min. The importance of speeding up the reaction is particularly important for the high pH part of the curve, since no degradation took place during the short periods the wool had to be in contact with alkali. Desorption of acid cannot be speeded up to the same extent and the washing procedure described is necessary.

Rate of dyeing curves. The wool was fixed to a frame revolving at 300 rev./min. and the exhaustion of the dye bath determined colorimetrically (Alexander & Hudson, 1950).

The alkali solubility of the wool was determined by the method of Harris & Smith (1936). The load-extension measurements were made as described by Speakman (1947) on a modified fibre balance.

RESULTS

Esterification with alcohols catalysed by mineral acids

Effect of concentration of acid. Simple kinetic considerations suggest that the rate of esterification is proportional to the concentration of hydrogen

ions which for strong acids is the same as the concentration of acid. From Fig. 1 it can be seen that this relationship appears to hold approximately for ethylation with hydrochloric acid as the catalyst. Analogous results were obtained with methanol and *n*-butanol, the only other alcohols which were examined in detail. Concentration of acids greater

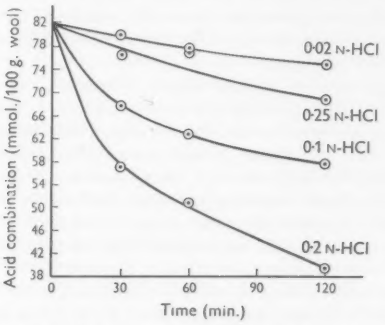

Fig. 1. The effect of different concentrations of HCl in ethanol containing 5% (v/v) of water on the rate of esterification of wool at 78°.

than 0·2N could not be used since these begin to hydrolyse the wool at the temperatures used. Sulphuric acid in concentrations of 0·1N or lower is as effective as hydrochloric acid of the same normality, but at higher concentrations the catalytic effect of sulphuric acid is less, probably because it is incompletely ionized at low pH values. The reversible formation of alkyl hydrogen sulphate may also contribute to the decreased activity of sulphuric acid at high concentrations.

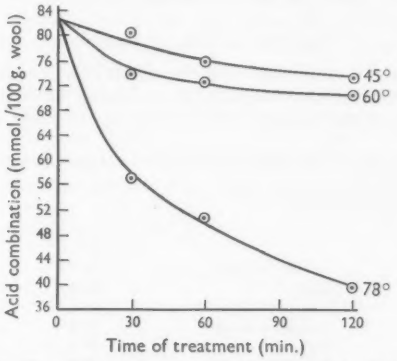

Fig. 2. The effect of temperature on the rate of esterification of wool by ethanol containing 2% of water and 0·2N-HCl.

Effect of temperature. The rate of esterification is dependent on temperature, and at 25° it was found that only 10 and 30% of the carboxyl groups had reacted with ethanol containing 0·1N-hydrochloric acid in 1 and 6 days respectively. With methanol the reaction was somewhat faster and under the same conditions 55% of the carboxyl groups were esterified in 6 days.

In Fig. 2 the rates of ethylation at three temperatures are compared. The great increase in rate on raising the temperature from 60 to 78° is, in all probability, due not only to the speeding up of the reaction but also to the increased rate of removal of water from the wool by the vigorous agitation which occurs at the boiling point.

The influence of water. From Table 1 it can be seen that there is no significant difference in the rate of methylation on changing from a completely anhydrous medium to a solution containing 1% of water, but that with 5% of water there is a very significant decrease.

Table 1. *Effect of water on rate of esterification of wool at 60° with methanol containing* 0·1N-HCl

Water content (%, v/v)	Acid combined (m-equiv./100 g. wool) after 0·5 hr.	1 hr.	2 hr.
0	56	40	31
0·6	54	37	32
1	52	36	33
5	64	41	—
8	78	69	61

Esterification with different alcohols. Fig. 3 shows the rate of reaction of different alcohols under completely anhydrous conditions either at 100° or at the

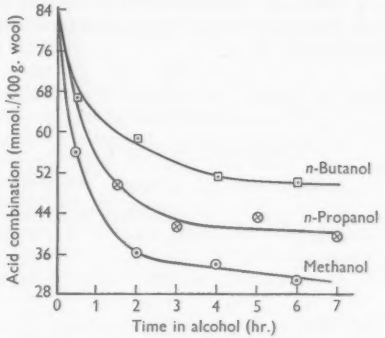

Fig. 3. Rate of esterification of wool with the following anhydrous alcohols containing 0·1N-HCl; methanol at 65°, *n*-propanol at 97°, *n*-butanol at 100°.

boiling points of the alcohol. In every case the curve flattened out after 4 hr. and after 6 hr. all the carboxyl groups capable of reacting had been esterified. In this way the proportion of carboxyl groups which

are available for esterification by alcohols of varying molecular weights and complexities were determined (see Table 2).

Table 2. *Maximum esterification of wool at* 100° *with different anhydrous alcohols in the presence of* 0·1 N-HCl *or at the boiling point of the alchohol, if below* 100°

(Time of reaction 6 hr. In every case minimum acid-combining value was reached after 4 hr. of reaction, cf. Fig. 3.)

Alcohol	Acid-combining value of wool (m-equiv./100 g. of wool)	Proportion of total carboxyl groups available for esterification (%)
Methanol	28	67
Ethanol	36	56
n-Propanol	38	54
*iso*Propanol	70	15
n-Butanol	50	39
*iso*Butanol	65	21
sec.-Butanol	70	15
n-Amyl alcohol	65	21
Ethylene glycol	40	51
Glycerol	44	46
Pentane-1:5-diol	65	21
Benzyl alcohol	72	12
Cetyl alcohol dissolved in xylene	72	12

The value for the maximum amount of esterification (i.e. for the minimum acid-combining value) for each alcohol was independent of the concentration of acid, but increased slightly in the presence of 0·5 % of water in the alcohol (see Table 3). With more than 2 % of water the rate of the reaction was found to decrease sharply and the wool after reaction was seen to be degraded, probably owing to acid hydrolysis.

Table 3. *Effect of water content of alcohol on maximum esterification of wool*

(Conditions as in Table 2.)

Alcohol	Water content (%, v/v)	Acid-combining value of wool (m-equiv./100 g. of wool)
n-Propanol	0	44
	0·5	39
	2	41
n-Butanol	0	57
	0·5	50
	2	53

The esterification can, of course, be formulated as an equilibrium

$$\text{Wool}-\text{COOH}+\text{ROH}\rightleftharpoons\text{Wool}-\text{COOR}+\text{H}_2\text{O},$$

and it might be considered that the reason why only 40 % of the carboxyl groups can be esterified with *n*-butanol as opposed to 70 % with methanol is not related to accessibility of carboxyl groups in the wool, but arises from different equilibrium constants in the two cases. However, if the extent of the reaction were governed by equilibrium considerations, the addition of 0·5 % of water to the butanol would reduce the number of carboxyl groups esterified to less than 1 %. This is not the case, and it can therefore be concluded that steric factors in the wool are responsible for the differences observed between the alcohols.

Hydrolysis of peptide bonds. Fraenkel-Conrat & Olcott (1945) showed that no hydrolysis of peptide bonds occurred with 0·1 N-hydrochloric acid in methanol at room temperature. The following experiments indicate that there was no substantial peptide bond breakdown in wool under anhydrous conditions when the same reagent was used at 65° for 4 hr., 70 % of the carboxyl groups being esterified. (1) The ester groups were readily saponified by dilute ammonia and the original acid-combining power was restored. If main chain degradation had taken place new carboxyl groups would have been formed, the acid-combining value being increased. (2) The work necessary to stretch a fibre in acid when salt links are broken was not significantly changed on esterification (see Table 10). (3) The solubility of the wool in alkali was only increased from 11 to 13 % on esterification. Both the elastic properties and the solubility in alkali are very sensitive tests for main chain degradation and the small changes produced by the treatment can probably be ascribed to the blocking of the carboxyl groups.

If the reaction is not carried out under anhydrous conditions, peptide bond hydrolysis occurs, the solubility in alkali is increased and the work to stretch a fibre in acid decreased.

Esterification with 1:2-*epoxides*

Table 4 shows that of the epoxides examined only epichlorhydrin is capable of esterifying more than 10 % of the available carboxyl groups in wool. The reaction was complete after 2 hr. in every case and a lower acid-combining figure than that given in

Table 4. *Combination of wool with acid after treatment with different epoxides in* 5 % *(w/v) aqueous solution at* 95° *for* 2 *hr.*

Substance used	Acid-combining value (m-equiv./100 g. of wool)
None	81·6
Ethylene oxide	81·4
Propylene oxide	77·4
Glycidol	78·7
*iso*Eugenol oxide	75·6
Styrene oxide	72·0
Epichlorhydrin	51·0

Table 4 was not obtained with any epoxide even after very prolonged treatment. These results are in contrast with those of Fraenkel-Conrat (1944) who found that ethylene oxide, propylene oxide and epichlorhydrin are equally reactive with globulins. Differences in the rate of hydrolysis of the epoxides in aqueous solution cannot explain the great superiority of epichlorhydrin as an esterifying agent for the carboxyl groups in wool, since in every case at least 50% of the epoxide originally employed was still present at the end of the experiment. Though the epoxides combine at different rates with organic acids (Brønsted, Kilpatrick & Kilpatrick, 1929), epichlorhydrin does not stand out in the order of reactivities and there is no correlation with the results obtained on wool.

The effect of temperature and of concentration of epichlorhydrin on the extent of the reaction is seen from Table 5. An esterification of approximately

Table 5. *Effect of concentration of epichlorhydrin and of temperature on rate of esterification of wool*

Concentration of epichlorhydrin (%, w/v)	Temp. (°)	Acid combined (m-equiv./100 g. of wool) after 0·5 hr.	1 hr.	2 hr.
5	95	—	52	51
	70	—	54	54
1	95	73	67	64
	70	80	74	70
0·3	95	78	75	75
	70	—	77	75

40% (corresponding to an acid-combining value of 51 m-equiv./100 g.) seems to be the maximum attainable with epichlorhydrin, as a higher value was not obtained under any experimental conditions.

In distilled water the pH of the epichlorhydrin solution is approximately 6 and this remained sensibly unchanged throughout the reaction. Experiments in which the solutions were adjusted with sulphuric acid and phosphate buffers to different pH values showed that the optimum pH range was between 5 and 7 for reaction with carboxyl groups.

Extensive reaction with groups other than carboxyl was indicated from a weight increase of 9–10% on treatment with epichlorhydrin. The cystine content of the wool was unchanged and this proved that the disulphide bond had not taken part in the reaction. After hydrolysis the concentration of the basic amino-acids and tyrosine was the same as that of untreated wool. From this it cannot be concluded that the free amino and phenolic groups did not react with epichlorhydrin, since compounds would be hydrolysed by the treatment with acid. The setting power of wool is reduced when the amino groups have been reacted with 1:2:4-fluorodinitrobenzene (Farnworth & Speakman, 1948). After reaction with epichlorhydrin wool could no longer be given a permanent set (Table 6), whereas after esterification with alcohols the setting power of fibres is increased (Blackburn & Lindley, 1948). This indirect evidence indicates reaction of the epoxide with amino groups which was shown to occur readily with soluble proteins by Fraenkel-Conrat (1944). In view of the results of Fraenkel-Conrat (1944), reaction of epichlorhydrin with phenolic groups in the wool is probable; but this could not be proved, since phenolic groups cannot be determined in unhydrolysed wool.

Saponification of esterified carboxyl groups

It was found that saponification was complete in less than 1 hr. in 0·1N-sodium hydroxide at room temperature, but no saponification occurred even after prolonged contact with distilled water at 100°. Washing esterified wool under tap water of pH approx. 7·8 caused some saponification after extended periods (Table 7). The rate of saponification of methylated wool in 0·1N-ammonia and a soap solution is shown in Fig. 4.

Although the rate of esterification of different alcohols decreased with increasing molecular weight, there was no corresponding difference in the rate of saponification. The esters formed with the different alcohols examined were readily saponified, although the esters containing groups of greater molecular weight appeared to react somewhat more slowly. However, it is doubtful whether these differences are significant (Table 8).

The esters are much more stable to acid than to alkali and there was no indication of hydrolysis after 3 days in 0·1N-hydrochloric acid at room tempera-

Table 6. *Effect of epichlorhydrin treatment on setting power of single wool fibres. Fibres held stretched 30% for 1 hr. in water at 100°*

Treatment	Percentage retained after releasing fibres in water at 100° for 2 min.	15 min.	30 min.	60 min.
None	19·5	17·8	17·0	16·4
	18·3	13·7	11·1	9·0
Epichlorhydrin treated (42 m-equiv./100 g. of wool)	8·6	1·5	−0·7	−4·5
	7·7	2·8	0·3	−2·1

ture. The esters are, however, hydrolysed under conditions used for hydrolysing peptide bonds in wool (i.e. 5N-hydrochloric acid at 120°).

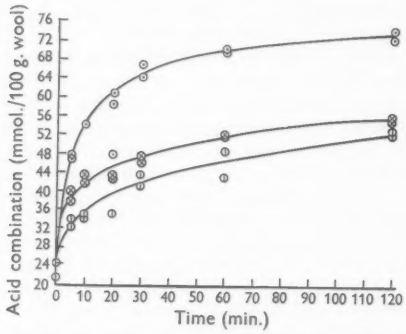

Fig. 4. Rate of saponification of wool which had esterified with methanol (0·1N-HCl) in the following solutions: 0·1N-NH_3 at 60°, —⊙—; 0·1N-NH_3 at 25°, —⊗—; 0·5% soap solution at 60°, —ⓘ—.

Table 7. *Hydrolysis of methylated wool on keeping for different periods under running tap water*

Time under tap water (hr.)	Acid-combining value (m-equiv./100 g. of wool) Exp. 1	Exp. 2
6	—	31
9	25	—
18	28	42
54	46	60
135	71	75

Effect of alkali on wool treated with epichlorhydrin

Wool which had been esterified with epichlorhydrin and then reacted with alkali was found to combine very much more slowly with acid, even under conditions of violent agitation, than untreated wool, while wool esterified with alcohols, whether it has been in contact with alkali or not, as well as epichlorhydrin-treated wool which has not been in contact with alkali, showed maximum acid combination after 4 min. (see p. 630) and no further acid was consumed on standing. It seemed possible that the slow additional acid uptake of wool esterified with epichlorhydrin and then treated with alkali results from the following series of reactions:

Table 8. *Hydrolysis of different esters of wool by treatment with* 0·1N-NH_3 *at* 60° *for* 30 *min.*

Substance used for esterification	Acid-combining value of wool (m-equiv./100 g.) Before hydrolysis	After alkaline hydrolysis
(Untreated wool)	81·7	82·1
Methanol	32·6	70·4
	40·7	72·5
Ethanol	50·6	71·8
	70·0	78·1
n-Propanol	49·0	72·5
*iso*Propanol	68·7	78·2
n-Butanol	58·1	72·8
Ethylene glycol	61·5	74·9
Pentane-1:5-diol	73·7	79·2
*iso*Eugenol oxide	75·6	81·2
Styrene oxide	72·0	82·0
Benzyl iodide*	50·7	68·6
Cetyl iodide†	49·8	81·9

* pH 8 phosphate buffer containing 5% (v/v) benzyl iodide shaken with wool for 2 hr. at 95°.
† 5% solution (w/v) in *iso*propanol for 2 hr. at 70°.

In agreement with this mechanism it was found that between 80 and 90% of the calculated quantity of chloride ions were liberated on treating epichlorhydrin-treated wool with 0·1N-ammonia at 60° for 3 min. Moreover, experiments with the model substance, α-monochlorhydrin, showed that ring formation was complete with 0·1N-sodium hydroxide in less than 2 min., whereas complete acid hydrolysis of the epoxide in 0·1N-hydrochloric acid at 25° required 5 hr.

It was found (Table 9) that after contact for only 4 min. with dilute alkali such as 0·02M-borax or 0·1N-ammonia, the acid-combining value of epichlorhydrin-treated wool after 20 hr. in acid was increased to that of untreated wool although no such increase occurred after 4 min. in acid. From these results it can be concluded that little saponification occurred, but that the probable epoxide formation was complete during the 4 min. reaction with alkali.

Hence, the difference between the quantity of acid consumed by epichlorhydrin-treated wool after 4 min. and after 24 hr. was taken as a measure of the number of epoxide groups in the wool.

The rate of saponification of epichlorhydrin-treated wool is seen from the rate of increase in the

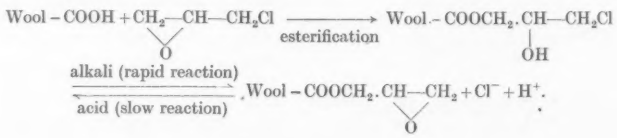

Table 9. *Formation of epoxide groups in wool treated with epichlorhydrin by reaction with alkali*

Alkali used	Time of treatment at 60° (min.)	Acid-combining value (m-equiv./100 g. in acid) for 4 min.	Acid-combining value (m-equiv./100 g. in acid) for 20 hr.	m-equiv. of epoxide in 100 g. wool
0·1 N-NH_3	0	53·0	53·4	0·4
	2	65·4	79·6	14·2
	10	77·0	85·5	8·5
	30	80·0	86·5	6·5
	60	82·7	86·9	4·2
0·05 M-Borax	0	53·6	53·9	0·3
	2	62·6	82·3	19·7
	10	69·9	81·7	11·8
	30	73·7	84·8	11·1

4 min. acid-combining value and the number of epoxide groups present from the difference of the two acid-combining values. By short treatment with alkali virtually complete conversion to epoxide can be obtained without hydrolysis of the ester. The rate of saponification of epichlorhydrin-treated wool is, within experimental error, the same as that of wool esterified with simple alcohols (Table 9).

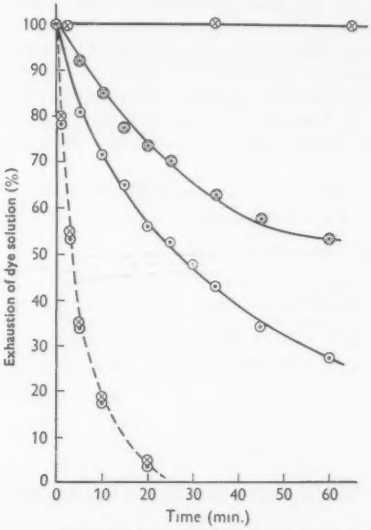

Fig. 5. Rate of dyeing of esterified and ordinary wool in a solution containing 200 mg. of azo geranine 2GS (Colour Index 637) only (——) and 250 mg. of Orange II and 0·1 N-HCl (– – – –); temperature 25°; solution to wool ratio 150 : 1; wool rotated at 200 rev./min. —⊗—, untreated wool; —⊡—, wool esterified with methanol (acid-combining power 46 m-equiv./100 g.); —⊙—, wool esterified with methanol (acid-combining power 25 m-equiv./100 g.).

Properties of esterified wool

Dyeing with simple acidic dyes takes place by an ion-exchange process in which the inorganic anion held by a charged amino group is replaced by the dye anion of greater affinity (Alexander & Kitchener, 1950) and can only take place when some carboxyl groups are masked. In ordinary wool anion exchange is possible only in acid solutions, when the carboxyl groups have combined with a proton, but when some of the carboxyl groups are esterified the corresponding amino groups retain a positive charge in solutions of pH less than 10 and carry a 'Gegenion' which can take part in ion exchange. In agreement with this concept it is seen from Fig. 5 that in neutral solution only the esterified wool takes up dye, whereas in acid solution both esterified and untreated wool dye equally readily. Basic dyes such as methylene blue are bound as 'Gegenions' to charged carboxyl groups and as a result it is found that esterified wool at all pH values takes up much less of these dues than untreated wool.

Esterification of the carboxyl groups with alcohols results in the same increase in the wash-fastness of dyes on wool which Schlack (1947) found after reaction with epichlorhydrin. Simple acidic dyes sorbed from acid solution lose affinity for the wool in neutral solutions when the ionized carboxyl groups neutralize the amino groups, but the slow diffusion of the big dye ions makes the diffusion out of the fibre a slow process. In esterified wool, however, the carboxyl groups do not compete with the dye anions for the amino group and the wool only loses its affinity for the dye anion in alkaline solution when the amino groups lose a proton and become un-ionized.

The titration curve of methylated wool is shown in Fig. 6, and the combination of the unblocked carboxyl groups with acid occurs at the same pH and is affected by neutral salt in the same way as in untreated wool. The combination of alkali with esterified wool, however, is shifted to lower pH values and shows a smaller influence of salt, probably because the amino groups no longer form part of a salt linkage and can now titrate as free base. Since combination with alkali is complete in 2 min. at room temperature no saponification occurs during the titration at pH less than 12. The acid-combina-

tion curve of wool treated with epichlorhydrin is exactly the same as that of methylated wool.

The work necessary to stretch wool fibres in acid solution is less than that in neutral solutions, and the difference is thought by Speakman & Hirst (1933) to represent the contribution of the salt links

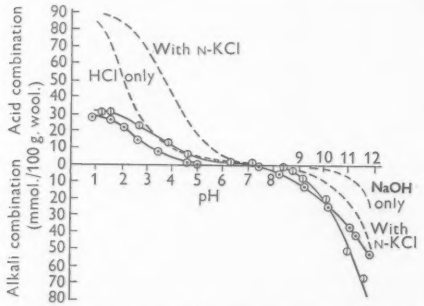

Fig. 6. Titration curve of ordinary wool (– – – –) and wool esterified with methanol (0·1 N-HCl) (——). —⊙—, titrated in a solution containing acid or alkali only; —ⓘ—, titrated in a solution containing N-KCl.

to the strength of the fibre. In Table 10 it is seen that this difference is decreased on esterification and that the reduction is directly related to the decrease in acid-combining power.

The esterification of carboxyl groups by amyl alcohol and butanol adds further proof that the concept of a maximum pore size put forward by Speakman is erroneous and that the dry wool fibre is accessible to large molecules although the rate of penetration is slow. From consideration of the kinetics of diffusion of both molecules and ions from aqueous solutions, Alexander, Gough & Hudson (1949*b*) showed that a model involving micropores containing free liquid was untenable and suggested that the wool fibres behaved as a homogeneous gel. The fact that the number of carboxyl groups available for esterification by alcohols varies with the molecular weight of the latter requires a modification of this picture. It is interesting that not all the carboxyl groups in wool are accessible even to the small molecule, methanol, which was shown by Fraenkel-Conrat & Olcott (1945) to esterify completely a number of proteins. The reason why alcohols of high molecular weight are capable of esterifying more carboxyl groups in the presence of low concentrations of water (Table 3) can either be due to the fact that increased swelling makes more groups accessible or that increased degradation by acid (see p. 632) leads to an opening of the structure. The failure of epoxides other than epichlorhydrin to react with the carboxyl groups in wool, an effect not observed with the proteins studied by Fraenkel-Conrat (1944), also requires elucidation since

Table 10. *The difference in the work needed to stretch a fibre in a phosphate buffer at* pH 7 *and in* 0·1 N-HCl *after different degrees of esterification*

(Work required for untreated wool in pH 7 buffer taken as 100.)

Treatment	Acid-combining value (m-equiv./100 g.)	Work required to stretch fibres 30% in		Difference in work to stretch in buffer at pH 7 and 0·1 N-HCl (%)
		Buffer at pH 7	0·1 N-HCl	
None	81·6	100	59·8	41·2
Epichlorhydrin	54·0	80·5	58·5	32·0
Methylation	44·4	76·7	57·6	28·6
	73·3	91·6	58·0	37·0

DISCUSSION

Speakman (1930, 1931) deduced from the elastic properties of fibres in different alcohols that wool is not accessible to molecules larger than propanol, but is readily penetrated by methanol and ethanol. He also concluded that in the presence of water the fibre can be penetrated by much larger molecules than in the absence of water. King (1947) showed that alcohols of higher molecular weight than ethanol are capable of diffusing into the wool fibre, but that the diffusion coefficients are so small that no penetration would be detected at room temperature in the period given by Speakman.

steric factors are obviously not operative in this case.

An explanation for the differences in accessibility may be found in the recent separation of wool into a fraction of high molecular weight and low cystine content and a fraction of low molecular weight and of high cystine content (Alexander & Earland, 1950). Preliminary investigations show that the low molecular weight fraction is exceptionally rich in acidic amino-acids. This low molecular weight fraction acts as a cement between micelles made up of the high molecular weight molecules and the carboxyl groups in the two phases would, of course, vary in accessibility.

The rate of hydrolysis of esters in solution is highly dependent on the nature of the alcohols, e.g. hydrolysis of methyl acetate is twice as fast as that of ethyl, *n*-propyl and *n*-butyl acetate and six times as fast as that of *iso*propyl acetate (Smith & Olsson, 1925). The fact that the different wool esters hydrolyse at approximately the same rate suggests that the rate-controlling step in the heterogeneous reaction may not be the actual hydrolysis but a diffusion process.

SUMMARY

1. The effect of concentration of acid catalyst, temperature and water on the esterification of the carboxyl groups in wool with alcohols has been studied.

2. The number of carboxyl groups capable of being esterified decreases with increasing molecular weight of the alcohol.

3. The esters formed are readily saponified by alkali and the rate of this reaction is independent of the alcohol used.

4. Of a number of 1:2-epoxides examined only epichlorhydrin was found to esterify more than 10% of the carboxyl groups of wool. The ester of monochlorhydrin formed reacts with dilute alkali at room temperature to form an epoxide which is combined in the wool.

5. The effect of esterifying the carboxyl groups on the titration curve, dyeing behaviour and elastic properties of wool have been investigated.

6. It is shown that the combination of acids with wool can be greatly speeded up by efficient agitation and that equilibrium can be established in a few minutes.

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Blood Glutathione and Non-glucose Reducing Substances in Diabetes

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A relation between glutathione and experimental diabetes was first suspected when Leech & Bailey (1945) observed a simultaneous fall in blood alloxan and glutathione following the injection of diabetogenic doses of alloxan into rabbits. Lazarow (1946, 1947) subsequently demonstrated that glutathione and other sulphydryl compounds such as cysteine and BAL (2:3-dimercaptopropanol) were capable of protecting the rat against the diabetogenic activity of alloxan. More recently Conn, Louis & Johnston (1949) have shown that the intravenous administration of reduced glutathione inhibited the temporary diabetes produced by the administration of adrenocorticotrophic hormone (ACTH).

It is possible that the great variation observed by Mosenthal & Barry (1946) in the non-glucose reducing

substances in the blood from diabetics might be explained by variations in the concentration of glutathione in the blood and that in turn these might be related to the type or severity of diabetes. Previous investigations concerning the concentration of glutathione in the blood of diabetic patients have given conflicting results. Thus, while Campanacci (1930) and Varela, Apolo & Vilar (1930) found low levels, Platt (1931) and Dogliotti & Meloni (1935) found the blood levels in diabetic patients to be the same as in normal individuals. More recently, Stock & Currence (1950) have determined the concentration of reduced glutathione in the blood of 14 fasting diabetic patients and observed no significant difference from the normal range. This paper reports similar investigations on 39 diabetic patients, under somewhat different conditions from those studied by Stock & Currence, by a method which determines both reduced and oxidized glutathione. At the same time, we have compared the concentration of glutathione in the blood with the difference between the 'true' and 'apparent' blood-sugar concentrations.

CLINICAL MATERIAL

The 39 subjects studied were all ambulatory diabetic patients attending the out-patient diabetic clinic at King's College Hospital. Some attending for routine checks had relatively normal blood sugars; others, some of whom have not received insulin treatment, had high blood sugars and showed ketosis. Blood samples were taken by venepuncture into a mixture of fluoride and oxalate at 10 a.m., in most cases about 2 hr. after breakfast. A series of 20 normal healthy medical students and laboratory staff were investigated under similar conditions.

METHODS

All estimations were performed in duplicate. 'True' blood sugar was determined by the Harding & Downs (1933) modification of the Schaffer-Hartmann (1921) method performed on $CdSO_4$-NaOH filtrates (Fujita & Iwatake, 1931). 'Apparent' blood sugar was determined by the same method using Folin & Wu tungstic acid filtrates. The nitroprusside reaction for glutathione was always negative when performed on the $CdSO_4$ filtrates, but was strongly positive in all the tungstic acid filtrates. The concentrations of reduced and total glutathione in the blood were determined by the method of Woodward & Fry (1932) on sulphosalicylic acid filtrates of whole blood prepared within 10 min. of venepuncture.

RESULTS

Table 1 presents the glutathione levels observed in the 20 normal individuals, in 18 diabetics without ketosis, and in 21 diabetics who had heavy ketosis and whose urine gave positive tests for acetoacetic acid with ferric chloride.

Table 1. *Glutathione levels in blood of normal and diabetic patients*

Group	No. of cases	Reduced glutathione (mg./100 ml.) Mean ± S.E.M.	Reduced glutathione (mg./100 ml.) Range	Total glutathione (mg./100 ml.) Mean ± S.E.M.	Total glutathione (mg./100 ml.) Range
Normal	20	33·5 ± 0·82	25·6–40·5	43·0 ± 0·88	36·8–50·5
All diabetics	39	30·1 ± 0·74	20·4–43	39·1 ± 0·81	27·3–53
Diabetics without ketosis	18	32·1 ± 1·18	23·6–43	41·1 ± 1·22	32·2–53
Diabetics with ketosis	21	28·5 ± 0·81	20·4–36·8	37·5 ± 0·93	27·3–45·4

	Comparison of groups	t	P
Reduced glutathione	Normals/diabetics	3·1	<0·01
	Normals/ketosed diabetics	4·1	<0·01
	Normals/non-ketosed diabetics	0·99	0·4 –0·3
	Ketosed diabetics/non-ketosed diabetics	2·5	0·02–0·01
Total glutathione	Normals/diabetics	3·26	<0·01
	Normals/ketosed diabetics	4·3	<0·01
	Normals/non-ketosed diabetics	1·26	0·3 –0·2
	Ketosed diabetics/non-ketosed diabetics	2·4	0·02–0·01

Table 2. *Correlation of blood-sugar differences with total glutathione levels*

(Difference = 'Apparent' − 'True' blood sugar.)

Group	No. of cases	Mean blood sugar difference (mg./100 ml.)	Range	Mean blood glutathione (mg./100 ml.)	r*	P
Normals	7	19·0	15–23·5	43·1	+0·422	0·1
Ketosed diabetics	21	26·9	16–52	37·5	+0·072	0·1
Non-ketosed diabetics	18	24·6	2–39	41·1	+0·217	0·1

* r is the correlation coefficient.

Total glutathione. The values for total glutathione in the whole group of diabetics were significantly below normal. While the concentration of total glutathione in the diabetics without ketosis was within the normal range, a significantly lowered concentration was observed in those with ketosis, and accounted for the significant difference found between our normal values and those of the whole group of diabetic cases.

Reduced glutathione. Table 1 also shows that the changes in reduced glutathione paralleled those of total glutathione.

Non-glucose reducing substances. Table 2 presents the mean values of the difference between the 'true' and 'apparent' blood-sugar concentration in the same diabetic patients. Statistical analysis shows that there is no correlation between this difference and the total glutathione concentration in the blood either for normal or diabetic subjects.

DISCUSSION

The reduction in the blood glutathione concentration in diabetes is in agreement with a recent report of Binkley, Fujii & Kimmel (1950), whose normal values, however, differ from those we have observed. This difference may be because Binkley *et al.* used a method for the determination of glutathione depending on hydrolysis to cysteine which was then estimated by the Sullivan & Hess (1936) method. Our results for normal subjects, which agree with those of previous workers (Platt, 1931; Stock & Currence, 1950), were obtained by the iodometric method. Binkley *et al.* found in diabetes that the lowered value of glutathione was compensated by an increase in the concentration of γ-glutamylcysteine. Since this substance would be estimated as glutathione by the iodometric method, a fall in blood glutathione due to its conversion to γ-glutamylcysteine would therefore not be detected by this method. The question of the specificity of the methods of estimation of glutathione clearly requires further investigation in the light of the results of Binckley *et al.*

The complete lack of correlation between total blood glutathione and the non-glucose reducing substances demonstrates clearly that in addition to glutathione other non-glucose reducing substances must be present in variable quantities in the blood. One case showed this very clearly because the blood glutathione was 33 mg./100 ml., equivalent to 8·6 mg. glucose/100 ml. by the Schaffer-Hartmann method, and yet the difference between the 'true' and 'apparent' blood sugar was only 2 mg./100 ml. The discrepancy is beyond any experimental error and shows that the blood must have contained a non-glucose reducing substance other than glutathione which passed into the cadmium filtrate to be estimated with the 'true' glucose. Recently Fraser (1950) has shown that the ergothioneine concentration is significantly higher in diabetics than in normals, and it is therefore possible that the above results may be due to an increase in the ergothioneine concentration or some other unknown substance in this particular patient.

The decrease in glutathione concentration in the blood could be due to one or more of several factors. There is no published evidence of an increased glutathione excretion in diabetes and it has not been possible for us to investigate this point. It is possible that the fall in glutathione in diabetic ketosis is due either to decreased production or to increased requirements for sulphydryl compounds. Blood glutathione, however, is contained almost entirely within the red cells and it is therefore more probable that the fall in glutathione content of the blood is associated with some change occurring within the red cells. Grunert & Phillips (1949) have shown that sodium deficiency is accompanied by a low level of blood glutathione in the rat. Binet & Pautonet (1943) have reported that the low serum sodium found in patients with Addison's disease is accompanied by low levels of blood glutathione which are restored to normal by the administration of deoxycorticosterone acetate. Diabetic ketosis is known to be associated with an increased elimination of sodium and it is possible, therefore, that the concomitant fall in blood glutathione is brought about directly by sodium deficiency. This clearly requires further investigation.

SUMMARY

1. Total and reduced blood glutathione have been determined in 20 normal and 39 diabetic persons. A significant reduction in glutathione was found only in the diabetics with severe ketosis. This reduction may be related to the reduction of base in diabetic ketosis.

2. The glutathione content of blood has been compared with the difference between the concentration of 'true' glucose and total reducing substances. Glutathione does not always account for this difference, which suggests that other reducing substances not yet identified must be present in the blood.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **290th Meeting** *of the Biochemical Society was held in the National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7, on Friday, 20 October, 1950, when the following papers were read:*

COMMUNICATIONS

The Relationship between Growth Response of *Lactobacillus leichmannii* 313 and Aeration of the Stationary Assay Medium. By K. A. Lees and J. P. R. Tootill (introduced by W. B. Emery). (*Glaxo Laboratories Ltd., Fermentation Division, Barnard Castle, County Durham*)

Details of a microbiological tube assay for vitamin B_{12} employing *Lb. leichmannii* 313 A.T.C.C. 7830 as test organism have previously been published (Lees & Emery, 1949).

During the routine application of this method it became evident that the variable response of the test organism to standard doses of vitamin B_{12} could, to a large extent, be explained by difference in the aeration of the test medium and the presence of reducing substances in the samples being assayed.

Our investigations into the relationship between the growth of *Lb. leichmannii* 313 and the oxygenation of the medium have established that there is a significant linear regression of growth on surface area of medium exposed to the atmosphere. This regression accounted for all the variation not attributable to experimental error.

On the assumption that dissolved oxygen and consequent inhibition of growth would decrease asymptotically to a finite value with increasing depth, mathematical analysis leads to the expectation of a regression of growth response on the reciprocal of medium depth at sufficiently large depths. We have confirmed this expectation for depths of medium equivalent to 8 ml. (and upwards) of medium in 6 × 1 in. assay tubes.

Cooper & Woodman (1946) published a formula for the diffusion of antiseptics through agar gels, and from this we have derived the following formula appropriate to our experimental conditions, assuming that similar principles of diffusion hold for the diffusion of oxygen through stationary assay medium as for the diffusion of substances through agar gels, and that the inhibition of growth is proportional to the dissolved oxygen content:

$$g = A - \frac{B}{V}\int_0^{KV} e^{-\frac{1}{2}K^2V^2}\,d(KV),$$

where g = mean growth determined by log turbidity, V = volume in 6 × 1 in. tubes (proportional to depth), and A, B and K are empirical constants.

The experiment was then re-analysed applying this formula (the constants being estimated from the experimental data), and the results showed that the formula accounted for all the relevant variation encountered.

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Further Observations on the Stimulation by Insulin of Fat Synthesis by Lactating Mammary Gland Slices. By Judith H. Balmain and S. J. Folley. (*National Institute for Research in Dairying, University of Reading*)

As has been pointed out (Folley & French, 1949, 1950), lactating mammary tissue is a particularly favourable medium for the *in vitro* study of fatty acid synthesis, for mammary slices will metabolize acetate alone (ruminants) or in presence of glucose (non-ruminants) with R.Q. > 1. The conclusion that, under these conditions, the high R.Q. indicates net fat synthesis received strong support from an experiment on a lactating goat demonstrating the rapid incorporation of intravenously injected ^{14}C-labelled acetate into milk fat (Popják, French & Folley, 1950).

Accordingly, following rather inconclusive reports suggesting that insulin might stimulate the incorporation of acetate into fatty acids by rat liver slices (Bloch & Kramer, 1948; Bloch, 1948), we were able, using lactating rat mammary slices, to demonstrate a marked stimulation by insulin of fat synthesis from glucose + acetate (Balmain, French & Folley, 1950). We have now further studied this effect,

particularly in relation to the physiological state of the mammary gland, in experiments on Norway rats and sheep, using methods as previously described except that the respiration measurements were made over 2 hr. instead of 3 hr.

With glucose (0·3 %) + acetate (0·02 M) as substrate, addition of crystalline insulin (Novo) had no effect on $-Q_{O_2}$, Q_{acid} or R.Q. of rat tissue (11 rats) taken shortly before parturition (20 days' pregnancy). By contrast, the marked effects of insulin, previously observed, on the much more active respiratory metabolism of fully lactating tissue (15 days post-partum: 11 rats) were fully confirmed. Tissue from 10 rats weaned for 2 days at the 20th day of lactation, though showing somewhat higher respiration than before parturition, was also insensitive to insulin. Similar differences in insulin sensitivity at the three stages were shown with 0·3 % glucose as substrate though, as before, the effect of insulin on $-Q_{O_2}$, and R.Q. of fully lactating tissue, was smaller than when acetate was also present. The close relationship of insulin to the acetate metabolism of fully lactating rat mammary tissue (15 days) is shown by the results in Table 1, which are calculated from mean values obtained from experiments on 11 rats.

The above results indicate that insulin by itself is incapable of stimulating fat synthesis in fully developed but essentially non-functional mammary tissue, even though some milk fat can probably be synthesized before parturition (Popják, Folley & French, 1949); its effect seems to depend on the existence of an active secretory condition presumably related to the action of anterior-pituitary hormones, perhaps needing regular application of the suckling stimulus for their continued secretion.

Table 1. *Influence of insulin on the metabolism of rat mammary tissue*

(Δ signifies differences between values in glucose (0·3 %) + acetate (0·02 M) and in glucose (0·3 %) alone.)

	$\Delta - Q_{O_2}$	ΔQ_{acid}	Δ R.Q.
Insulin	+3·7	−8·0	+0·28
No insulin	+0·7	−4·7	+0·02

Sheep mammary slices showed no *in vitro* response to insulin at any stage of lactation studied (11–48 days) either in acetate alone or in acetate + glucose, this possibly constituting a further metabolic difference between mammary tissue of ruminants and non-ruminants (see Folley & French, 1950).

Addition of glycerol (0·2 %) to rat mammary slices metabolizing glucose + acetate gave effects similar to insulin but less marked save in the case of Q_{acid}. Glycerol was somewhat less effective than insulin in raising the glucose uptake. With lactating sheep tissue the glycerol effect was observed with acetate (0·02 M) as substrate, but not with glucose + acetate. Further work on the effect of glycerol in this system is being done to clarify any possible relationship between the action of glycerol and the mechanism of the insulin effect.

We are indebted to Dr K. Hallas-Møller of Novo Terapeutisk Laboratorium, Copenhagen, for supplies of insulin free from glycogenolytic factor.

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The Effect of Vitamin B_{12} on the Conversion of Glycine to Choline. By H. R. V. Arnstein and A. Neuberger. (*The National Institute for Medical Research, Mill Hill, London, N.W.* 7)

In an earlier communication one of us (Arnstein, 1950) reported that methanol, formate, the β-carbon atom of L-serine, the α-carbon atom of glycine are precursors of choline methyl groups in the rat. Similar findings have recently been obtained by various American investigators. The results to be reported now are concerned with the quantitative significance of this formation of methyl groups from non-essential dietary constituents under various conditions.

Albino rats were put on the experimental diets soon after weaning. In diets A, B, C, D and E the nitrogen requirements were provided by an amino-acid mixture (18·11 % of the diet) containing the essential amino-acids with the exception of methionine which was replaced by homocysteine. The diets F and G contained 12·5 % casein. All the basal diets contained the known vitamins including folic acid (2 mg./kg. diet), with the exception of vitamin B_{12} and choline. Vitamin B_{12} concentrate (equiv. to 2·5 μg./rat/day) was added to diets A, B, D and F, choline (5 mg./rat/day) was added to diets B and C, and DL-methionine (25 mg./rat/day) was added to diets D and E. All the diets contained

2 % ^{14}C-methylene-labelled glycine (56·8 μc./mol.). After 24, 35 or 39 days the animals were killed; the two rats in each group were combined and glycine was isolated chromatographically as the crystalline dinitrophenyl derivative separately from the carcass and internal organs, whilst choline from the two sources was combined and degraded.

The two rats on diet A, which is devoid of labile methyl groups but contains vitamin B_{12}, grew at a very slow rate (0·13 g. and 0·64 g./day respectively), whilst the rats which received 5 mg. choline but no vitamin B_{12} (diet C) lost weight steadily. The rats receiving vitamin B_{12} and 5 mg. choline (diet B) grew moderately well (0·68 and 1·2 g./day respectively). The growth rate of the rats receiving the amino-acid diet and 25 mg. methionine/day was not markedly affected by the presence of vitamin B_{12}. The results of the radioactivity measurements are shown in Table 1.

Table 1. *Specific radioactivities of substances isolated* (μc./mol.)

	Choline			Glycine	
Diet*	Total	Ethanolamine moiety	Trimethylamine moiety	(internal organs)	(carcass)
A (basal amino-acid + vitamin B_{12})	30·73	17·60	13·13	21·29	14·73
B (basal amino-acid + vitamin B_{12} + 5 mg. choline/day)	18·28	12·57	5·71	25·12	18·74
C (basal amino-acid + 5 mg. choline/day)	10·10	8·40	1·70	23·15	14·32
D (basal amino-acid + vitamin B_{12} + 25 mg. methionine/day)	19·90	15·95	3·95	24·66	15·90
E (basal amino-acid + 25 mg. methionine/day)	18·65	15·61	3·04	24·66	15·82
F (casein + vitamin B_{12})	22·87	19·63	3·24	23·16	21·27
G (casein)	29·49	23·05	6·44	24·28	21·17

* The specific activity of the glycine fed was 56·8 μc./mol.

It can be seen that the activities of the carcass and organ glycine are similar in animals which had grown moderately well, whilst in animals which had lost weight or grown poorly, the activity of the carcass glycine is much lower than that of the organ glycine even after 39 days. A comparison of the activity of the fed glycine with that isolated from the internal organs allows an approximate calculation to be made of the extent of endogenous glycine formation. The estimates from the various experiments agree and suggest that this is about 1·5–2·0 mM/100 g. body wt./day.

The activity of the ethanolamine moiety of choline is similar to that of the glycine except in the animals which had received choline in their diet. The relative activities of the two carbon atoms of ethanolamine were not determined, and the extent to which ethanolamine is derived from glycine can therefore not be accurately calculated. The activity of the trimethylamine moiety is low on diets lacking vitamin B_{12} (diet C) or where methionine has been provided (diets D and E). The activity in the methyl groups is particularly high in diet A (presence of B_{12} and absence of dietary methyl groups); the effect of vitamin B_{12} is very marked if the results for diets B and C are compared.

Recent American work (e.g. Stekol, Bennett, Weiss, Halpern & Weiss, 1950) has provided evidence on the basis of feeding experiments that vitamin B_{12} is concerned with the synthesis of methyl groups. Our present results strongly support this conclusion.

The authors wish to acknowledge the gift of a vitamin B_{12} concentrate from Dr W. F. J. Cuthbertson (Glaxo Laboratories Ltd.).

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The Thyroxine-Inhibitory Action of a Series of Ethers of *n*-Alkyl 3:5-Diiodo-4-hydroxybenzoates. By J. H. Wilkinson, Mary M. Sheahan and N. F. Maclagan. (*Department of Chemical Pathology, Westminster Medical School, London*)

Following the observations that the *n*-alkyl 3:5-diiodo-4-hydroxybenzoates (Wilkinson, Sheahan & Maclagan, 1950) exhibited a definite antithyroxine action when tested by the oxygen consumption method in mice (Maclagan & Sheahan, 1950), the effect of a series of related ethers was studied. Frieden & Winzler (1949) reported the antithyroxine effect of the benzyl ether of 3:5-diiodo-

4-hydroxybenzoic acid in tadpoles. This compound was shown by us to have moderately weak activity in mice, its methyl and ethyl esters being active only at a total dosage of 1000 mg./kg. The methyl, ethyl and *n*-propyl ethers of 3:5-diiodo-4-hydroxybenzoic acid were shown to be active at 400 mg./kg., whilst the ethyl esters of the methoxy and ethoxy compounds displayed activity at 100 and 50 mg./kg. respectively. Esterification of 3:5-diiodo-4-*n*-propoxybenzoic acid, however, diminished its activity, the methyl ester being active at 1000 mg./kg. and the ethyl ester inactive at this dosage.

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The Relative Metabolic Inertia of Collagen in the Rat. By J. C. PERRONE (*British Council Scholar*) and H. G. B. SLACK. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Five rats (initial weight range 205–237 g.) were fed, over a period of 3 days, glycine labelled with ^{14}C in the methylene group. Each rat received 50 mg. glycine containing 20 μc. radioactive glycine/200 g. body wt. The rats were killed after 1, 7, 14, 21 and 34 days. Liver and muscle (from all four limbs) were removed and freeze-dried. The white tendons from all four limbs, tail and back were dissected out and cleaned from muscle and connective tissue. Collagen was obtained free from other proteins by a modification of the method of Bowes & Kenton (1948). The free glycine from liver and muscle was removed by repeated extraction with boiling 75 % (v/v) ethanol. The glycine present in collagen and in the mixed proteins of liver and muscle was obtained after hydrolysis as crystalline 2:4-dinitrophenyl derivative using chromatography on buffered Celite columns (Perrone, unpublished work). The results are shown in Table 1.

The extremely low activity of the glycine in the collagen indicates that the turnover of this protein is exceedingly slow, very much slower than the mixed proteins of muscle. A more quantitative interpretation is difficult owing to the fact that these animals were still growing, weight increases up to 46 g.

Table 1. *Radioactivities (counts/min./mg. sample) of samples of* 2:4-*dinitrophenylglycine prepared from the muscle, liver and collagen of rats which had received* $H_2N^{14}CH_2.COOH$

	Time after administration (days)				
	1	7	14	21	34
Collagen	46·05	68·0	50·7	127·6	151·0
Muscle	698	603	422	454	383
Liver	3700·3	1414	550	286	148

having been observed during the experiment. The possibility therefore exists that collagen is in fact completely inert and that the observed radioactivity is entirely due to the deposition of new collagen. The limitations of the concept of the dynamic equilibrium of proteins have recently been emphasized by Sprinson & Rittenberg (1949).

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Studies on the Metabolism of the Circulating Erythrocyte in the Rabbit. By HELEN M. MUIR, J. C. PERRONE (*British Council Scholar*) and G. POPJÁK. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

A normal adult rabbit was injected with

$NH_2.{}^{14}CH_2.COOH$ and $CH_3.{}^{14}COONa$

containing 57 and 100 μc. of ^{14}C respectively. At intervals 10 ml. blood were removed and the red-cell cholesterol and protoporphyrin isolated. Glycine, obtained as the 2:4-dinitrophenyl (DNP) compound, was isolated from the hydrolysate of the total red-cell protein by chromatography on a buffered Celite column. The radioactivities of the three components are given in Table 1.

The results show that the cholesterol of the cell membrane is metabolically active, unlike the porphyrin of haemoglobin, where the isotope content

remains unchanged during the life of the cell (Shemin & Rittenberg, 1946; for the rabbit, see Neuberger & Niven, 1949). The radioactivity of the porphyrin can therefore be used to show the extent of blood regeneration following losses in sampling.

Table 1. *Radioactivity of red-cell constituents (counts/min./mol.)*

Time after injection	Cholesterol	Proto-porphyrin	Proto-porphyrin ÷8	Glycine DNP
6 hr.	247	29·45	3·68	5·67
24 hr.	318	132·6	16·58	19·4
72 hr.	288	240·5	30·05	35
7 days	219	281·2	35·15	31·1
22 days	93	228·5	28·55	26·9
52 days	27	105	13·12	13·42

The half-life of the cholesterol was found to be 12–14 days, the radioactivity changing in an approximately logarithmic fashion, whereas the activity of the porphyrin fell off linearly to half its value in 39 days. The rapid turnover of red cell cholesterol whose isotope concentration reached a maximum within 24 hr. and then decreased rapidly, has also been demonstrated in a goat and another rabbit where loss of blood was negligible. Cholesterol may be regenerated by a synthetic mechanism, or by exchange with plasma cholesterol, as has been shown for phospholipin (Hahn & Hevesy, 1939; Zilversmit, Entenman, Fishler & Chaikoff, 1943).

Eight glycine molecules are used in the synthesis of protoporphyrin (Muir & Neuberger, 1950; Wittenberg & Shemin, 1950). Hence, by dividing the molecular activity of protoporphyrin by eight, the radioactivity of the glycine used for its synthesis can be calculated, and is found to be similar to that of the glycine in the protein. This might suggest that protein and porphyrin are formed simultaneously. However, Thorell (1947) considers from spectroscopic and cytological evidence that the protein is formed first. The present results do not necessarily contradict those of Thorell, because the preformed protein might remain metabolically so active that complete exchange with body glycine takes place at the time the porphyrin is synthesized. After the porphyrin is attached to the protein the decline of the metabolic activity of each should be similar.

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A New Method for the Chemical Degradation of *n*-Fatty Acids. By G. D. Hunter and G. Popják. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

In the studies of biosynthesis of fatty acids with carbon isotopes it became of importance to locate the position of labels in the fatty acids in order to obtain information about their mode of synthesis.

It is more than a century-old observation that oleic acid, when fused with KOH, yields palmitic and acetic acids (Varrentrapp, 1840). Apparently under the influence of molten KOH the double bond in oleic acid migrates from the 9:10 to the α, β position and then fission occurs.

We found similarly that hex-2-enoic acid when fused with KOH at 300–330° gives butyric and acetic acids in good yield.

The method described here aims at the preparation of the α, β-unsaturated fatty acid and has so far been carried out on hexanoic acid as a model. The steps involved are: α-bromination of the acid chloride, conversion of the brominated product to ethyl or higher ester and dehydrobromination of the bromo-ester with quinoline, yielding the ester of the Δ^2-ethenoid acid. When the ethyl or octyl ester of hex-2-enoic acid, thus prepared from hexanoic acids, was fused with KOH at 300–330° it gave butyric and acetic acids. These two products of degradation were identified by paper chromatography and were separated by azeotropic distillation. The overall yield of butyric and acetic acid, starting with 2 g. of hexanoic acid was about 30–35 %. Both acetic and butyric acids can be degraded further by familiar methods (see Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949).

When synthetic [1-^{14}C] hexanoic acid was thus degraded, only the carboxyl carbon of acetic acid carried the label, indicating that no shift of the carbon atoms of the acid occurred during the degradation.

This method is likely to prove successful with higher fatty acids also. So far it has been used for the degradation of hexanoic acid obtained from the milk

of a lactating goat after the injection of carboxyl-labelled acetate (cf. Popják, French & Folley, 1950); the results are described in the following communication.

Our thanks are due to Dr J. W. Cornforth for suggesting the possibility of the method and for his advice during the investigation, and to Miss Joan Webb for helping us with the chromatographic identification of the degradation products.

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Mode of Formation of Milk Fatty Acids Studied with the Aid of $CH_3{}^{14}COONa$.

By T. H. French,* G. D. Hunter, A. J. P. Martin and G. Popják. (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

In a previous communication the utilization of acetate for the synthesis of milk fatty acids in a lactating goat was reported (Popják, French & Folley, 1950). It was found that after the injection of $CH_3{}^{14}COONa$, ^{14}C appeared rapidly in the milk glyceride fatty acids. The specific activities of the milk fatty acids indicated that synthesis in the udder from small molecules is of primary importance in the origin of milk fat and that the short-chain, steam-volatile fatty acids originate from the condensation of C_2 units.

The behaviour and relationship of the specific activity-time curves of four crude fatty acid fractions (cf. Popják *et al.* 1950), suggested that the differences in the specific activities of the crude fatty acid fractions were connected in some way with the biochemical mechanism of their synthesis rather than with any other factor (rate of synthesis, dilution with non-isotopic blood fat). To obtain information on this point, samples from certain periods of the experiment were pooled and fractionated into individual acids and their ^{14}C contents determined. The identity and purity of the acids was established by chromatography. Typical results are shown in Table 1. It is noteworthy that acetic acid has been identified and isolated for the first time as a component acid of mammalian fat. In addition traces of propionic and valeric acids were identified also.

Table 1. *Specific activities of pure fatty acids obtained from glycerides of goat milk after a single injection of 5 mc. of* $CH_3{}^{14}COONa$

(Specific activities are expressed as 1×10^{-3} μc. ^{14}C/mg. fatty acid carbon.)

	Period of experiment from which acids isolated	
Fatty acid	0–48 hr.	12–48 hr.
Acetic	11·80	—
Butyric	7·63	0·350
Hexanoic	9·86	0·424
Octanoic	—	0·717
Decanoic	—	0·943
Dodecanoic	—	1·080
Tetradecanoic	—	1·260
Palmitic	—	1·260
Stearic	—	0·063
Oleic	—	0·063

Table 2. *Specific activities of individual carbon atoms of acetic, butyric and hexanoic acids of goat milk fat after the injection of* $CH_3{}^{14}COONa$

(Specific activities are expressed as 1×10^{-3} μc. ^{14}C/mg. C)

	1 (COOH)	2	3	4	5	6	Average of all C atoms
Acetic	23·6	0·00	—	—	—	—	11·8
Butyric	15·26	0·00	15·26	0·00	—	—	7·63
Hexanoic	32·60	0·00	13·35	0·00	13·35	0·00	9·86

Typical results of chemical degradations of acetic, butyric and hexanoic acids are shown in Table 2. These show that butyric acid is synthesized by the condensation of two C_2 units of equal isotope content and that hexanoic acid is formed by the addition of a C_2 unit to a C_4 compound and not by the simultaneous condensation of three C_2 compounds. The results show quite clearly that carbon atoms 3 and 5 of hexanoic acid correspond to the carboxyl- and β-carbon atoms of butyric acid and therefore the elongation of the C_4 to the C_6 acid occurred by the addition of a C_2 unit to the carboxyl end of the shorter acid.

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The stepwise elongation of the fatty acids, C_4 to C_6, C_6 to C_8, etc., explains satisfactorily the increasing specific activities of the fatty acids with increasing chain length. Thus it appears that the short-chain fatty acids represent intermediates in the synthesis of the longer ones, and by the process of rapid secretion into the milk have escaped elongation.

Stearic and oleic acids in the milk either originate from the blood, or their primary precursor is not acetate.

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Liquid-Gas Partition Chromatography. By A. T. James and A. J. P. Martin. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

The ratio of vapour pressures of two members of a homologous series is usually greater than the ratio of partition coefficients between two liquid phases. Therefore, a gas-liquid partition chromatogram of similar efficiency to a liquid-liquid partition chromatogram should lead to a better separation of the two compounds (cf. Claesson (1946), for studies on gas adsorption analysis). This has been found to be the case for the volatile fatty acids using a chromatogram containing a static liquid phase and a mobile gas phase of nitrogen. This method should be applicable to a very wide range of substances, possibly all those that can be distilled without decomposition, and possesses the advantage that the lower limit of quantity used is determined only by the efficiency of detection. In general, substances emerge from the column in the order of their boiling points except in cases where the energy of association with the stationary solvent differs markedly from the energy of association of the pure substances. This is illustrated by the series of amines, ammonia (b.p. $-33{\cdot}5°$), monomethylamine (b.p. $-6{\cdot}5°$), trimethylamine (b.p. $3{\cdot}5°$) and dimethylamine (b.p. $7{\cdot}4°$) where the order of emergence is ammonia, tri-, mono- and di-methylamines, using an undecanol-diethanolamine mixture as the solvent.

The apparatus consists of a 1·2 m. length of 4 mm. internal diameter glass tube ending in a short length of capillary, that fits into a titration cell. The chromatogram tube is enclosed in a vapour jacket and contains kieselguhr as the support for the liquid phase. The mixture of substances is applied at the open end of the tube and nitrogen is applied to the tube at constant head. The substances are titrated in the cell as they emerge from the column. Temperatures have been used such that the partial pressures of saturated vapour of the substances fell between 10 and 100 mm. The following acids have been separated in 0·5 mg. quantities, formic, acetic, propionic, *n*- and iso-butyric, *n*- and iso-valeric, *n*- and iso-hexanoic, heptanoic and octanoic acids using as the stationary solvent liquid paraffin-stearic acid or d.c. 550 silicone with acid washed Celite 545 as the carrier.

The technique was demonstrated.

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Enzymic Decarboxylation of 2:3-Dihydroxyphenylalanine. By H. Blaschko and H. Langemann. (*Department of Pharmacology, Oxford*)

Racemic 2:3-dihydroxyphenylalanine was prepared from 3-benzamido-2-keto-8-methoxycoumarin by treatment with hydriodic acid and red phosphorus (Harington & McCartney, 1927). Carbon dioxide was formed when the amino-acid was incubated anaerobically with guinea pig kidney extract. The formation of the corresponding amine, 2:3-dihydroxyphenylethylamine, was demonstrated on the arterial blood pressure of the spinal cat: the extracts after incubation with the amino-acid had a pressor action when injected intravenously. The blood pressure effect resembled that of tyramine rather than that of adrenaline.

The amino-acid was also decarboxylated by an acetone-dried preparation of *Streptococcus faecalis* R.

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The Biological Activity for the Rat of the Bound Form of Nicotinic Acid Present in Maize. By E. KODICEK. (*Dunn Nutritional Laboratory, University of Cambridge, and Medical Research Council*)

In a recent communication Chaudhuri & Kodicek (1950) reported that a concentrate of the bound form of nicotinic acid prepared from wheat bran did not cure nicotinic acid deficiency induced in rats by a diet low in protein and containing a high proportion of maize (Harris & Kodicek, 1950). Similarly, small doses of wheat, rice or barley bran, although containing adequate amounts of bound nicotinic acid, had no curative effect. However, on alkaline digestion which liberated free nicotinic acid from its bound form, the cereals and the concentrate were fully active in curing the nicotinic acid deficiency. From microbiological assays Clegg, Kodicek & Mistry (unpublished) obtained evidence that maize, like other cereals, contains nicotinic acid in bound form.

The present experiments show that this form of nicotinic acid in maize is also unavailable to the rat. When rats deficient in nicotinic acid were given a diet in which the maize was replaced by maize hydrolysed with N-NaOH, their growth rate was about equal to that of control rats receiving an equivalent amount of nicotinic acid.

A specimen of zein, supplied by Glaxo Ltd., contained a high amount (100 μg./g.) of nicotinic acid, in bound form, as determined by chemical and microbiological assay. When rats were given a diet containing 5 % of this zein, 35 % maize meal and 3·5 % casein, the usual nicotinic acid deficiency was produced. When the zein was hydrolysed with N-NaOH, however, good growth was obtained similar to that of control rats receiving an equivalent amount of nicotinic acid. In contrast, a hydrolysate of another brand of zein, from Corn Products Ltd., which contained no nicotinic acid, did not improve the growth of nicotinic acid-deficient rats. The addition of histidine, valine, threonine, arginine and lysine, which have been reported by Kligler & Krehl (1950) to supplement the deficient protein, had no beneficial effect.

It thus appears that maize, in common with other cereals, contains a form of nicotinic acid which, unless previously hydrolysed by alkali, is unavailable to the rat. The pellagra-producing maize can be changed by alkaline digestion into a pellagra-preventive foodstuff. This is most likely due to the liberation of free nicotinic acid from its bound form, but others factors may also be involved. The implications of this finding were discussed.

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Histidine α-Deaminase of Mammalian Liver. By D. A. HALL. (*Department of Medicine, University of Leeds*)

Since Jaffe isolated urocanic acid from the urine of a single dog in 1874, α-deamination has been repeatedly proposed as a hypothetical primary reaction in the metabolism of histidine by the mammalian liver. However, Edlbacher, in a series of papers finally discussed in a review in 1943, claimed to have proved that although α-deamination was a possible step in histidine metabolism, there was no evidence that it was a normal one.

Previous difficulties have lain in the insensitive methods of identifying and estimating urocanic acid especially in the presence of histidine. An identification of the compound based on the marked increase in ultraviolet absorption produced by the introduction of a third conjugated double bond during the change from histidine to urocanic acid, has enabled μg. of the latter to be demonstrated in the presence of mg. of histidine. The method has not been made fully quantitative owing to the rapid decrease in absorption observed on standing. This is most probably due to the photo-catalysed transformation from the *trans* to the *cis* form (Edlbacher & Heitz, 1943).

Identification of urocanic acid among the metabolic products of histidine after incubation with enzyme preparations from liver, enables the concept of α-deamination to be placed in its correct perspective, as the initial stage of one of two possible routes by which histidine may be degraded to glutamic acid.

The deaminase is present in rat, cat and rabbit liver, but has not been encountered in other tissues examined. The production of urocanic acid is proportional to the concentrations of both substrate and enzyme. The pH optimum is in the region of 7·8.

Crude enzyme preparations are obtained by precipitation of an aqueous or phosphate buffer extract of whole liver with two volumes of cold acetone, to give a dry powder with an average nitrogen content of 14·6 %. This powder can be stored at 0° without loss of activity, but urocanic acid, which is itself inhibitory, is produced from the histidine present in the preparations on standing, and this lowers the effective activity of the enzyme. The urocanic acid can be removed by dialysis to restore the activity of the fresh preparation, any coenzyme being apparently stable to this treatment.

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The Component Fatty Acids of the Faecal Lipids of Normal and Cholesterol-Fed Rats. By D. C. Edwards and R. P. Cook. (*Department of Biochemistry, University College, Dundee*)

The faecal non-volatile fatty acids of rats, fed a basal diet of rat cake (North-Eastern Agricultural Co-operative Society) without and with 16·6 % olive oil, in the absence and presence of 1·7 % cholesterol, have been fractionated. A fraction insoluble in light petroleum (b.p. 40–60°) was removed initially and consisted apparently of degradation products of cholesterol (Cook, Polgar & Thomson, 1950). The light petroleum-soluble fraction was fractionated by Twitchell's method and the methyl esters of the solid and liquid acids fractionally distilled *in vacuo* (Hilditch, 1940).

The excretions of the main acid fractions on a kg. body wt./day basis are shown in Table 1. In the presence of cholesterol there is an increase in total acids excreted. On the low fat diet the increase was in liquid and light petroleum-insoluble acids. On the fat-containing diet there was an increase in solid, liquid and light petroleum-insoluble acids.

Table 1. *Component fatty acids of faecal lipids*

(Excretions expressed as g./kg. body wt./day.

C = control. S = + cholesterol.)

Diet		Lipid	Total acids	Solid acids	Liquid acids	Light petroleum-insoluble acids
Low fat	C	1·0	0·6	0·3	0·3	0·1
	S	2·5	0·8	0·3	0·4	0·2
+ Fat	C	0·9	0·6	0·2	0·3	0·1
	S	2·2	1·2	0·4	0·7	0·2

The percentage weight of the component light petroleum-soluble acids is shown in Table 2. The feeding of fat to the control animals altered the pattern of acids, the percentage of saturated acids being lessened and that of the unsaturated acids being increased. On the addition of cholesterol to the diets the pattern of the acids from the low fat diet was similar to those of the control series, and the faecal acids from the olive oil-cholesterol diet were similar to their control series.

Table 2. *Percentage weight of component faecal fatty acids*

(C = control. S = + cholesterol.)

		Saturated			
Diet		C_{14}	C_{16}	C_{18}	Residue
Low fat	C	4	24	7	10
	S	4	25	9	9
+ Fat	C	0	14	3	8
	S	3	14	6	7

		Unsaturated				
Diet		C_{14}	C_{16}	Oleic	'Linoleic'	Residue
Low fat	C	1	17	30	7	9
	S	6	14	21	0·5	18
+ Fat	C	0	19	43	6	12
	S	0	11	44	2	11

It would therefore appear that the addition of cholesterol to a diet causes an increased excretion of normal non-volatile fatty acids, but their pattern is little different from normal. The relation of the excretion to the esterification of cholesterol is as yet uninvestigated.

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The Faecal Volatile Acids of Normal and of Cholesterol-Fed Rats. By R. O. THOMSON. (*Department of Biochemistry, University College, Dundee*)

Two types of basal diet (*a*) 'Rat cake' (North-Eastern Agricultural Co-operative Society, Aberdeen) and (*b*) 'Synthetic', consisting of a mixture of known compounds and of low cellulose content, without and with 16·6 % olive oil, in the absence and presence of 1·7 % cholesterol, were fed to groups of male piebald rats. The faeces were collected and the lipid extracted with ether and with ethanol. After saponification and extraction of unsaponifiable material, the volatile acids were steam distilled by the method of Friedmann (1938). The mixed acids were separated by chromatography (Moyle, Baldwin & Scarisbrick, 1948).

The distribution of the acids as percentages of the total volatile acids is shown in Table 1. In Table 2 are given the faecal excretions of the volatile acids on a kg. body wt./day basis.

Large amounts of volatile acids are excreted on the control rat cake diet. These are lessened when fat is fed. The feeding of cholesterol on the low-fat diet increases the amount of volatile acids and the increase is mainly in the C_2 fraction.

On 'synthetic' diets there is an increase in the excretion of total volatile acids on both low fat and olive oil diets after the feeding of cholesterol and the increase is mainly in the C_2 and C_3 fractions.

Table 1. *Distribution of volatile acids as percentages of the total volatile acids*

(C = control. S = + cholesterol.)

Diet		C_2	C_3	C_4	C_5	C_6 and higher acids
Rat cake	C	36	30	18	7	8
	S	56	26	8	7	4
Rat cake + olive oil	C	43	33	12	6	6
	S	49	32	7	4	9
Synthetic	C	13	9	8	15	56
	S	30	13	9	11	37
Synthetic + olive oil	C	35	29	12	11	12
	S	34	31	12	10	12

It is possible that in the degradation, as in the synthesis of cholesterol, acetic acid (Bloch & Rittenberg, 1942; Little & Bloch, 1950) is concerned.

Table 2. *The faecal excretion of volatile acids in mg./kg. body wt./day*

(C = control. S = + cholesterol.)

Diet		Total volatile acids	C_2	C_3	C_4	C_5	C_6 and higher acids	Loss of cholesterol
Rat cake	C	110	40	33	20	8	9	—
	S	170	96	45	14	12	7	300
Rat cake + olive oil	C	70	29	22	8	4	4	—
	S	70	32	21	5	3	6	360
Synthetic	C	10	1·2	0·9	0·8	1·4	5·4	—
	S	20	4·3	1·8	1·3	1·6	5·1	100
Synthetic + olive oil	C	10	4·2	3·5	1·4	1·3	1·4	—
	S	20	6·0	5·5	2·0	1·7	2·0	190

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Preformed Vitamin A in Marine Crustacea. By E. BATHAM, L. R. FISHER, K. M. HENRY, S. K. KON and S. Y. THOMPSON. (*National Institute for Research in Dairying, University of Reading*)

Krill taken from the stomachs of northern baleen whales contains preformed vitamin A but only traces of carotenes active as precursors of vitamin A (see Table 1). This agrees with findings with krill from antarctic whales (Thompson, Ganguly & Kon, 1949; Kon & Thompson, 1949*a*) and with free-swimming northern krill (Kon & Thompson, 1949*b*), but is contrary to the findings of Wagner (1939).

The non-saponifiable residue of the oils of the catches of *Meganyctiphanes norvegica* (M. Sars) and *Thysanoessa raschii* (M. Sars) already described (Kon & Thompson, 1949*b*) and that obtained from the antarctic krill analysed by Kon & Thompson (1949*a*) were tested biologically on rats for vitamin A activity. Further tests were done with another batch of *Meganyctiphanes norvegica* obtained in

Table 1. *Vitamin A and carotenoids in marine crustacea*

(For method of measurement see Kon & Thompson, 1949*b*)

Species	Locality and date	Vitamin A i.u./g.	Vitamin A i.u./g. oil	Carotenoids Total μg./g.	Carotenoids Total μg./g. oil	Carotenoids β-carotene
Meganyctiphanes norvegica	Loch Fyne, Jan. 1950	26	835	70	2190	Trace
M. norvegica	Loch Fyne, Aug. 1950	40	600	112	1660	Trace
Thysanoessa raschii	Loch Fyne, Jan. 1950	76	1360	58	1040	Trace
T. raschii	Loch Fyne, Aug. 1950	32	520	31	508	Trace
Euphausia superba from stomach of Blue whale	Antarctic, Mar. 1950	2	91	22	1016	Trace
From 1 g. original material: Soft parts		0·1*	—	8·6*	—	Trace
From 1 g. original material: Eyes		1·2*	—	8·2*	—	Trace
From 1 g. original material: Exoskeleton less eyes		0·5*	—	9·0*	—	Trace
Meganyctiphanes norvegica from stomach of Fin whale	200 miles N.N.W. of Bergen, June 1950	1·5	188	23	2810	Trace
M. norvegica from stomach of Blue whale	40 miles N.W. of St Kilda, Aug. 1950	5·0	392	48	3780	Trace
Calanus finmarchicus	Loch Fyne, Jan. 1950	0·1	6	7	420	Trace
	Loch Fyne, Apr. 1950	0·03	2	18	970	Trace
	200 miles N.N.W. of Bergen, June 1950	0·2	3	57	4810	Trace

* i.u./g. original material.

January 1950 (see Table 1). The biological measurement indicated about half the potency derived from the $SbCl_3$ test or ultraviolet absorption. We are studying now the reasons for this discrepancy.

Euphausia superba Dana obtained and dissected on the spot during the 1949–50 antarctic expedition of the W.F. *Balaena*, further samples of *Meganyctiphanes norvegica* and *Thysanoessa raschii* from Loch Fyne, and various other crustacea were also studied. Some of the results are given in Table 1. Two-thirds of the vitamin A of *Euphausia superba* were concentrated in the eyes. It is noteworthy that on no occasion could we detect in *Calanus finmarchicus* (Gunnerus) more than traces of vitamin A.

This work was supported by a grant from the Development Commission. We are very much indebted to Mr C. E. Ash and to Mr R. M. Brachi of the W.F. *Balaena* for the samples of *Euphausia superba*. It is a pleasure to record the kind help and hospitality given to two of us (S.K.K. and S.Y.T.) by Mr A. J. Hojem at Blomvåg Hvalstasjon and by Captain H. Harneshaug in their catcher *Hval* 2. L.R.F. and S.K.K. wish to thank similarly Captain H. Jespersen for facilities at the Whaling Station of Scottish Whalers Limited at West Loch, Tarbert, Harris. We are most grateful to Mr E. Ford and the Millport workers for their generous help.

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Vitamin B_{12} and the Biological Value of Proteins. By K. M. Henry and S. K. Kon. (*National Institute for Research in Dairying, University of Reading*)

The connexion between high protein diets and vitamin B_{12} deficiency (Hartman, Dryden & Cary, 1949; Rubin & Bird, 1947) and other evidence (e.g. Charkey, Wilgus, Patton & Gassner, 1950) indicate that vitamin B_{12} is concerned in protein metabolism.

The biological value of a sample of casein, prepared in the laboratory and proved by chick tests not to contain the 'Animal Protein Factor', was measured by the method of Mitchell (Mitchell, 1923–4; Mitchell & Carman, 1926*b*) on six pairs of littermate male rats previously rendered vitamin B_{12}-deficient as described by Emerson (1949). The ether-extracted whole egg included in the diet in the

standardizing periods was from eggs of hens deficient in the 'Animal Protein Factor' (Black, Getty, Coates, Harrison & Kon, 1949). The other components of the diets contained no vitamin B_{12} and were supplemented with pure vitamins. One member of each pair of rats received daily, in addition, a solution of 0·2 μg. crystalline vitamin B_{12}.

The biological value of the casein was $82{\cdot}2 \pm 1{\cdot}58$ for the depleted animals and $89{\cdot}7 \pm 0{\cdot}78$ for those given vitamin B_{12}. The difference is statistically significant. The corresponding values for true digestibility were $99{\cdot}9 \pm 0{\cdot}38$ and $99{\cdot}3 \pm 0{\cdot}35$, this difference is not significant.

Though this result demonstrates that assimilation of nitrogen is less in the absence of vitamin B_{12} than with it, there is no proof that this relationship is specific. In fact, similar effects of withdrawal of essential nutrients were observed by Kon (1931) for 'vitamin B_2', by Mitchell & Carman (1926*a*) for sodium chloride, and by Mayfield & Hedrick (1949) for vitamin B_1 and riboflavin.

In view of the suggestion that vitamin B_{12} is linked with the synthesis of methionine (Oginsky, 1950), further experiments will be done with casein supplemented with this and other amino-acids.

We are indebted to Mr D. J. G. Black, Department of Agriculture, Reading University, for the eggs from depleted hens, and to Glaxo Laboratories Ltd. for the crystalline vitamin B_{12}.

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Effect of Antibiotics and Vitamin B_{12} on the Growth of Normal and 'Animal Protein Factor' Deficient Chicks. By M. E. Coates, G. F. Harrison, S. K. Kon, M. E. Mann and C. D. Rose. (*National Institute for Research in Dairying, University of Reading*)

These experiments were done to confirm the report of Stokstad & Jukes (1950) that pure aureomycin hydrochloride, given by mouth, stimulated the growth of chicks, and to compare the effect of aureomycin with that of other antibiotics more readily available in this country. Groups of 5–10 normal chicks and of chicks depleted of the 'animal protein factor' (A.P.F.) as described by Coates, Harrison & Kon (1949) were used. Aureomycin HCl, streptomycin $CaCl_2$ complex, procaine penicillin, polymyxin and nisin were first tested; all were added to the diets to the extent of 2·5 mg./100 g. Examen (Glaxo Laboratories, Ltd.) was used throughout as a source of vitamin B_{12}. The results are shown in Table 1. As polymyxin and nisin were considerably less effective than the other three antibiotics they were omitted from further tests and were replaced by terramycin HCl (Finlay, Hobby, P'an, Regna, Routien, Seeley, Shull, Sobin, Solomons, Vinson & Kane, 1950). The antibiotics were given to the extent of 1·25, 2·5 and 10 mg./100 g. diet. Although all caused an increase in growth (Table 2), this was not graded with the dose.

Penicillin and terramycin were as effective as aureomycin in stimulating the growth of normal chicks and of deficient chicks given vitamin B_{12}. The growth-stimulating effect of the antibiotics on deficient chicks without vitamin B_{12} was small and

Table 1. *Effect of antibiotics on the growth of chicks*

(Values represent body wt. in g. at 3 weeks (Group 1) or at 4 weeks (Groups 2 and 3))

			Antibiotic					
Group	Type of chicks	Diet	None	Aureomycin	Strepto-mycin	Penicillin	Nisin	Poly-myxin
1	Normal	Stock*	179	190	192	211	177	187
2	A.P.F.-deficient	A.P.F.-deficient†	100	106	112	137	99	107
3	A.P.F.-deficient	A.P.F.-deficient† + 2 μg. vitamin B_{12}/100 g.	166	240	238	220	189	200

* The diet of Olsson (1941) + 1 % veterinary cod liver oil.
† Coates, Harrison & Kon (1949).

Table 2. *Effect of antibiotics on the growth of chicks*

(Values represent body weight in g. at 4 weeks.)

Dose in mg./100 g.	Aureomycin			Streptomycin			Penicillin			Terramycin		
	1*	2	3	1	2	3	1	2	3	1	2	3
10	280	157	209	276	139	227	277	130	222	287	127	225
2·5	295	126	194	265	118	209	258	139	208	264	119	212
1·25	244	114	224	261	130	178	247	139	199	274	126	209

Diets alone 1, 230; 2, 116; 3, 181.

*1. Normal birds given Olsson (1941) diet.
2. Deficient birds given deficient diet.
3. Deficient birds given deficient diet + 2 μg. vitamin B_{12}/100 g.

erratic; penicillin produced the most consistent gains.

Total bacterial counts on gut contents of deficient chicks with and without supplements of vitamin B_{12} were neither uniformly depressed nor clearly related to weight gains.

We are much indebted to Lederle Laboratories Division, American Cyanamid Company, for aureomycin HCl, to Glaxo Laboratories Limited for Examen, procaine penicillin and streptomycin $CaCl_2$ complex, to the Wellcome Physiological Research Laboratories for polymyxin, to Messrs Fisons Ltd. Loughborough for nisin, and to Mr D. J. G. Black for the A.P.F.-deficient chicks.

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Ethanolaminuria, An Obscure Metabolic Defect Associated with a Case of Primary Hepatoma.

By C. E. Dent, D. I. Fowler and J. M. Walshe. (*Medical Unit, University College Hospital, London, W.C.* 1)

A ninhydrin-reacting substance giving a weak spot in the position taken by ethanolamine on two-way phenol/collidine-lutidine paper chromatograms has often been found in the urines from cases of severe liver disease (Dent, 1947, 1948). In these cases it has always occurred in the presence of high concentrations of many amino-acids. In a further study of liver disease one of us (J.M.W.) noted from one patient a constant output in the urine of a substance giving a similar spot of much greater intensity. In this urine no other significant change occurred in any of the amino-acid outputs, these combined findings being so far unique in our experience. The urine was from a woman of 45 with a primary hepatoma of at least 3 years' duration. She enjoyed reasonably good general health and all the usual liver tests showed normal function.

Paper chromatographic analysis of the urine confirmed that of the many substances in our collection, only ethanolamine matched the behaviour of the spot exactly on ordinary runs using phenol and collidine-lutidine. In addition, it resembled ethanolamine in that it was volatile in steam (with some decomposition) from an alkaline solution, its R_F in phenol was greatly slowed when run under acid conditions, it did not form a copper complex under the conditions of Crumpler & Dent (1949), and it was readily destroyed by periodate. For final confirmation of its identity a pure sample was isolated by displacement chromatography from a column of Zeokarb 215 (Partridge & Westall, 1949). After applying the urine to the column the less basic substances were removed by development with 0·25N-NH_4OH. The ammonia and ethanolamine were then displaced with 0·125N-NaOH. The ammonia was removed by evaporation of the solution at low temperature and the ethanolamine then converted into its di-*p*-nitrobenzoyl derivative. This had m.p. (uncorr.) 190–191·5°, mixed m.p. with the synthetic ethanolamine derivative 188·5–190·5°. Found C, 53·2; H, 3·6; N, 11·4%. Calc. for $C_{16}H_{13}N_3O_7$, C, 53·5; H, 3·6; N, 11·7 %.

The output of ethanolamine was determined assuming that the additional NH_3 given off from the

alkaline urine on addition of an excess of KIO_4 was all from ethanolamine (there was comparatively little serine and threonine in the urine). On this basis about 0·6–1·0 g./24 hr. was being excreted, this figure agreeing well with rough estimates from the strength of the ninhydrin colour given by the spot. Preliminary metabolic experiments were reported in which ethanolamine, ethanolamine precursors and various methyl donors, have been given by mouth.

We believe that the metabolic defect is closely linked to the tumour tissue, being either its cause or the consequence of it. We do not think it can be a non-specific sign of liver dysfunction.

We gratefully acknowledge the advice and help of Mr R. G. Westall in the use of ion exchange resins.

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Metabolism of Gentisic Acid. By R. Consden and Winifred M. Stanier. (*The Canadian Red Cross Memorial Hospital, Taplow, Maidenhead, Berks.*)

Meyer & Ragan (1948) reported that, after administration of gentisates to rheumatic fever patients, only about one-quarter was recoverable as gentisate from the urine and none could be detected in the blood. We have found, however, that about 90 % of administered gentisate is recoverable, as such, from the urine, that it is rapidly excreted, but that blood levels of 0·2–0·3 mg./ml. can be maintained by sufficiently high dosages. Traces are metabolized to gentisic acid conjugates, other phenolic conjugates, and free salicylic acid.

Gentisic acid in sera and urines was estimated spectrophotometrically, densities of suitable dilutions being measured at 320 mμ. (ϵ for gentisic acid = 3920, cf. Kapp & Coburn (1942)). Sera and urines were also examined by paper chromatography (cf. Bray, Thorpe & White, 1950) using as developer *n*-butanol in an atmosphere of ammonia. The position of the spots was revealed by their fluorescence in ultraviolet light (cf. Bate-Smith & Westall, 1950), as little as 0·1 μg. of gentisic acid being detectable in this way. Elution of the gentisic acid from chromatograms and measurement of the absorption at 320 mμ. of the eluates gave values in close agreement with those obtained with the whole urine.

Paper chromatograms of urine of rheumatic fever or Still's disease patients treated with gentisate, revealed gentisic acid only in strength. Traces of other fluorescent spots were present, too small in amount to permit accurate spectrophotometric estimation. One of these was salicylic acid; the others, which formed a complex zone near the origin, proved to be derivatives of gentisic acid and other phenols, since these were produced after acid hydrolysis of the eluted spots. Chromatograms of the sera showed only one phenolic spot, namely, gentisic acid. Extraction of the sera with ethyl acetate, to remove gentisic acid, followed by acid hydrolysis failed to liberate any phenolic substances.

In one investigation a rheumatic fever patient was given a total of 22·5 g. gentisic acid during 12 hr. in five equal doses at 3-hourly intervals. Urine was collected from the start of administration, and blood was taken before the first dose, 1 hr. before, and 12 hr. after, the final dose. It was found that 19·5 g. gentisic acid was excreted in the first 24 hr., whilst only 0·96 g. was excreted in the next 24 hr. Thus, 20·5 g. gentisic acid was recovered. The second sample of serum contained 0·306 mg./ml. gentisic acid, whilst none was found in the third.

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Methyl Cellosolve as a Developing Solvent in Paper Partition Chromatography. By A. E. BENDER. (*Research Department, The Crookes Laboratories Ltd., Park Royal, London, N.W. 10*)

The choice of solvents for paper chromatography of amino-acids is rather limited. The solvents most frequently used in two-dimensional chromatography are phenol and collidine. In view of the several objections to collidine listed by Bentley & Whitehead (1950) a substitute is desirable. Aqueous methyl cellosolve has been found to satisfy many of the requirements of a developing solvent and can replace collidine with advantage.

Methyl cellosolve (ethylene glycol monomethyl ether) is miscible with water in all proportions, and a mixture of 9 vols. of methyl cellosolve to 1 vol. of water was found to effect satisfactory separation of many amino-acids. The R_F values in this solvent were slightly higher than the corresponding values in collidine quoted by Consden, Gordon & Martin (1944). Satisfactory two-dimensional chromatograms were obtained using phenol followed by methyl cellosolve.

Commercial methyl cellosolve may be used without further purification and the finished chromatogram is as clean as the fresh paper. This is an advantage when the amino-acid spots are to be cut out for quantitative colorimetric estimation.

An advantage in using a solvent completely miscible with water is that there is no change in composition and no consequent alteration in R_F values at different temperatures. Even at the extremes of 0° and 30° the R_F values of all the amino-acids tested were constant.

Bentley & Whitehead (1950) modified the statement of Consden, Gordon & Martin (1944) that volatile solvents completely miscible with water give amino-acid bands too broad to be useful, to exclude ascending chromatograms. These workers ascribe the failure of descending chromatograms under these conditions to slow saturation of the atmosphere of the chamber with solvent and water vapour, during which time a preferential evaporation of the solvent takes place from the paper. This difficulty has been overcome by various devices (Goodall & Levi (1947); Karnovsky & Johnson (1949)). In the present work no special precautions were taken to avoid this risk and no difficulty was encountered. A beaker of 90 % methyl cellosolve was kept at the bottom of the chamber and the lid was closed after the papers had been inserted and the trough filled with solvent. Identical results both in R_F values and usefulness of spots were obtained with both ascending and descending chromatograms.

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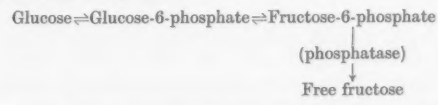
Phosphatase Activity in Fructose-Forming Tissues. By C. W. PARR and F. L. WARREN. (*Department of Biochemistry, University College, London*)

Free D-fructose has been shown to be present in the blood of foetal sheep (cf. Bacon & Bell, 1948). The site of origin of the fructose is located in the placental tissue (Huggett, Warren & Winterton, 1949; Huggett, Warren & Warren, 1950). Mann (1946) showed that the sugar in semen is D-fructose which is formed in the accessory male sex organs of many species.

The fructose of foetal blood appears to be derived from glucose, since increased blood concentrations of the latter sugar induce increased fructose production (Huggett *et al.* 1950). Mann & Parsons (1950) have also obtained evidence which indicates that glucose acts as a precursor of seminal fructose. It appeared probable that the pathway for the conversion might be:



Attempts have therefore been made to demonstrate the presence of a phosphatase capable of splitting fructose-6-phosphate (fructose-6-P) in fructose-forming organs. Most of the experiments so far have been carried out with rat accessory organs owing to the availability of this material at all seasons, but a few experiments, which will be

extended during the forthcoming gestation season for sheep, have shown similar results with placental tissue.

When fructose-6-P is added to buffered homogenates of rat accessory organ tissue there is a rapid conversion of some of the ester into glucose-6-phosphate (glucose-6-P) owing to the high phosphohexose isomerase activity of the homogenate. The equilibrium (about 30 % of fructose-6-P) is established within a few minutes. During incubation of the homogenate with this equilibrium mixture of fructose-6-P and glucose-6-P at 37° for 3 hr., free fructose is gradually liberated. The course of the liberation of fructose can be followed by removing samples at intervals and determining the fructose colorimetrically after precipitation with $Zn(OH)_2$ (a procedure which effectively removes fructose-6-P).

Analysis of such filtrates for total reducing sugars (T.R.S.) shows, however, that fructose accounts for only about 26 % of the T.R.S. It is assumed that the bulk of the non-fructose reducing sugar is glucose, since paper chromatography reveals a glucose spot of greater intensity than the fructose spot and no other sugar in like concentration. Confirmation is afforded by estimations of the inorganic phosphate liberated which show that the fructose formed is equivalent to about 25 % of the phosphate and thus 75 % of the latter is derived from some other dephosphorylation process.

The optimum pH for the formation of both fructose and T.R.S. is approx. pH 9·4. The enzyme responsible would thus appear to be an alkaline phosphatase and this is supported by the fact that it is significantly activated by Mg^{++} ions.

Mann & Parsons (1947) have shown that the fructose content of the accessory sex organs falls on castration and is restored after injection of testosterone. In conformity with this, homogenates of these tissues from castrated rats show reduced ability to liberate fructose from fructose-6-P, but the ability is restored after testosterone treatment. In this connexion it has been observed that the alkaline phosphatase activity (fructose-6-P substrate) of the kidneys of both male and female rats is reduced after castration.

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Fructose Formation and Seminal Phosphatase. By T. MANN and C. LUTWAK-MANN. (*Molteno Institute and the School of Biochemistry, University of Cambridge*)

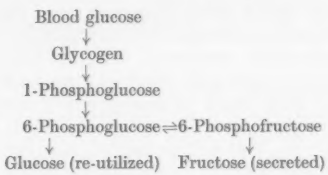
Fructose, the sugar of mammalian semen, originates in male accessory organs (Mann, 1946, 1949). The first indication that glucose may be the precursor of fructose was gained through *in vitro* experiments with bull seminal vesicle tissue (Mann & Lutwak-Mann, 1948); it was shown at the same time that unlike spermatozoa, accessory gland tissue glycolyses glucose but not fructose. Further, a strict dependence of seminal fructose upon blood glucose was demonstrated in experiments with alloxan-diabetic rabbits (Mann & Parsons, 1950). Recent studies concerned with the intermediary carbohydrate metabolism in accessory glands and seminal plasma gave the following results.

Fresh bull seminal vesicles contain up to 1 % glycogen which in minced tissue undergoes rapid amylolytic breakdown. However, dialysed extracts from bull seminal vesicle convert Cori ester through the action of phosphoglucomutase and phosphohexose isomerase to 6-phosphoglucose and 6-phosphofructose (ratio 2:1 to 3:1). These are dephosphorylated to glucose (estimated with glucose oxidase) and fructose (determined after removal of phosphohexoses with $Ba(OH)_2$ and $ZnSO_4$). If 6-phosphoglucose, 6-phosphofructose or 6-phosphomannose is acted upon by accessory gland extracts, the end product in each case is a mixture of free sugars. In the rat, analogous enzymic reactions have been demonstrated in the coagulating gland (main fructose-producing organ), but were also found in the seminal vesicle and ventral prostate. Castration caused considerable reduction, but no disappearance, of enzymic activity which was restored in rats treated with testosterone propionate.

Seminal plasma contains fructose (up to 1 % in bull and ram), but has no significant amounts of glycogen, glucose, phosphoglucose or phosphofructose. It dephosphorylates phosphohexoses *in vitro*. The phosphatase activity takes place at pH 7–9·8; the optimum pH is 9·3 (corresponding to alkaline phosphatase). Cori ester is dephosphorylated by seminal plasma to glucose owing to absence

of phosphoglucomutase. 6-Phosphoglucose and 6-phosphofructose yield a mixture of free fructose and glucose through the combined action of isomerase and phosphatase. Both phosphate groups are split off from 1:6-diphosphofructose, and free fructose accumulates together with a small amount of glucose. The same results were obtained with secretions collected directly from the accessory glands and with ejaculates from vasectomized bulls and rams.

Both *in vivo* and *in vitro* experiments suggest that the following enzymic reactions may lead to fructose accumulation in male accessory organs:



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The Decarboxylation of L-Malic Acid by *Lactobacillus arabinosus*. By P. M. Nossal. (*Department of Biochemistry, The University, Sheffield*)

Korkes & Ochoa (1948) have shown that *Lb. arabinosus* 17–5, when grown on media containing malic acid, forms an adaptive enzyme, 'malic decarboxylase', which converts L-malic acid to lactic acid and CO_2. This communication is concerned with the behaviour of washed suspensions of the organism towards L-malic acid. $Q_{CO_2}^{25°}$ (μl. CO_2 formed/mg. dry wt./hr.) on addition of malate was of the order of 10, but was increased to about 100 by yeast extract plus glucose. Of the known constituents of yeast extract, cozymase and a mixture of B vitamins were inactive, but $MnCl_2$ and KH_2PO_4 accelerated malate decomposition even more markedly than yeast extract. In the presence of $2{\cdot}5 \times 10^{-3}$M-glucose, $2{\cdot}5 \times 10^{-3}$M-$MnCl_2$ and 10^{-1}M-KH_2PO_4, $Q_{CO_2}^{25°}$ values rose up to 500, and addition of yeast extract had no further effect.

Table 1. *The effect of* K *ions on the rate of the decarboxylation of* L-*malate*

($1{\cdot}5$ mg. dry wt. cell suspension, $2{\cdot}5 \times 10^{-3}$M glucose, $2{\cdot}5 \times 10^{-3}$M-$MnCl_2$, $2{\cdot}5 \times 10^{-3}$M-sodium acetate buffer, pH 5·0, 20μmol. DL-malate. Total volume 4·0 ml. Gas space, air. 25°.)

KCl added (final concentration, M)	CO_2 produced in 30 min. (μl.)
None	36
$3{\cdot}2 \times 10^{-5}$	40
$1{\cdot}6 \times 10^{-4}$	78
$8{\cdot}0 \times 10^{-4}$	126
$4{\cdot}0 \times 10^{-3}$	154
$2{\cdot}0 \times 10^{-2}$	171
$1{\cdot}0 \times 10^{-1}$	186

Fructose, mannose, maltose and galactose, in decreasing order of effectiveness, acted like glucose, but L- and D-arabinose, D-xylose, D-ribose, sucrose, lactose, glycogen, lactate, cozymase and coenzyme II were inactive. Pyruvate accelerated malate decarboxylation, but less effectively than equivalent concentrations of glucose. Pyruvate itself was decomposed slowly to CO_2 and a substance giving the Voges-Proskauer reaction.

Adenosinetriphosphate, $Na_4P_2O_7$, $NaPO_3$ and NaH_2PO_4 failed to replace KH_2PO_4, which suggested that the active component of the latter was the K^+ ion. In fact, KCl was as effective as KH_2PO_4, and NaCl was inactive. Addition of NaH_2PO_4 in the presence of KCl caused no further increases in activity.

Potassium ions are known to be a component of the systems responsible for the reaction:

Pyruvate + adenosinetriphosphate
= phosphopyruvate + adenosinediphosphate

(Lardy & Ziegler, 1945); for the oxidative decarboxylation of malate to pyruvate by *Moraxella Lwoffi* (Lwoff & Ionesco, 1947); and possibly for the conversion of hexose monophosphate to hexose diphosphate (Munz, 1947). The optimum concentrations of K^+ ions for these reactions are 0·05, 0·01 and 0·01 M respectively. As can be seen from Table 1, the effectiveness of K^+ ions increases up to a concentration of 0·1 M in the malic decarboxylase system.

The optimum pH of the malate decarboxylation was 5·0 and the optimum temperature 35°. The half-maximum rate of decomposition was attained with a substrate concentration of $1{\cdot}8 \times 10^{-3}$M.

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Enzymic Method for the Estimation of L-Malic and Fumaric Acids. By P. M. Nossal. (*Department of Biochemistry, The University, Sheffield*)

Korkes & Ochoa (1948) have suggested the use of acetone powder extracts of L-malate-adapted cells of *Lactobacillus arabinosus* for the manometric estimation of malic acid. Washed suspensions of the organism were found to decarboxylate malic acid quantitatively if the solution contained glucose ($2{\cdot}5 \times 10^{-3}$ M), $MnCl_2$ ($2{\cdot}5 \times 10^{-3}$ M), KH_2PO_4 or KCl (10^{-1} M) and acetate buffer pH 5·0 ($2{\cdot}5 \times 10^{-2}$ M). 2 mg. dry wt. of cells decarboxylated up to 20 μmol. L-malic acid quantitatively in 1 hr.

Fumarate is not attacked significantly, but can be estimated as malate by the addition of a fumarase preparation (e.g. sheep's heart homogenate). The total CO_2 evolution is then a measure of malate plus fumarate. Succinate can similarly be determined by prior conversion into fumarate by the succinic dehydrogenase system.

In testing the specificity of the enzyme, it was found that of the substances likely to occur in biological material, only oxaloacetate and pyruvate interfered by also yielding CO_2. Oxaloacetate can be removed with aniline citrate, which does not affect the decarboxylation of malate. The decomposition of pyruvate is very slow at low concentrations so that if it is present in amounts below 10 μmol. per sample, malate can still be estimated by applying a small correction factor. Higher amounts of pyruvate can be removed by yeast carboxylase. Since this contains also fumarase (and thus alters the initial ratio of malate to fumarate in the sample) the following procedure is used. A portion of the sample is treated for 1 hr. with the complete *Lactobacillus* system, to remove malate. After 5 min. immersion in boiling water to inactivate the malic decarboxylase, 1·0 ml. of the carboxylase preparation of Krebs & Johnson (1937) is added to remove pyruvate. The standard method for malic acid applied to this sample gives the original fumarate content. Another portion of the sample is pretreated with carboxylase. Application of the standard malate method to this sample gives the quantity of fumarate plus malate.

Recovery of mixtures of malate and fumarate from pure solution and from aqueous extracts of biological materials and tissue homogenates was satisfactory.

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A Heat-Labile Vitamin B_{12} Complex in Faeces. By R. J. Pennington. (*Department of Physiology, Medical School, University of Birmingham*)

The *Lactobacillus lactis* Dorner (LLD) activity of filtered, warm-water extracts of rat faeces may be increased several-fold by heating to 100° or near. There is evidence that the effect is not due to heat-labile inhibiting substances, and the presence of a B_{12} complex, inactive for *Lb. lactis* Dorner, is suggested. Similar results were obtained with faeces from other animal species.

Pancreatin, papain, and takadiastase were without effect upon the activity of the unheated extracts.

The factor is presumably of bacterial origin, since the LLD activity of rat faeces greatly exceeded that of the diet. The activity was not appreciably decreased by the incorporation of succinylsulphathiazole in the diet.

The existence of a heat-labile, microbiologically inactive complex of B_{12} with a factor in gastric juice, and the probable existence of a similar complex in sera have been recently reported (Ternberg & Eakin, 1949; Ross, 1950). It thus appears probable that B_{12} forms several such complexes.

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DEMONSTRATION

Chromatographic Fractionation of the Water-soluble Constituents of Liver. By P. N. CAMPBELL. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Fresh pig liver cooled to 0° was homogenized in a blender and after adjustment of the pH to 5 the homogenate was poured into twenty times its volume of boiling water. The heat-precipitated protein was removed and the extract was dialysed. The dialysable material was fractionated on Zeokarb 225. The following ninhydrin-positive substances were provisionally identified as being absorbed on the column: glutathione, glutamic acid, glycine, alanine, valine, proline, phenylalanine and leucine. Approximately 40 % of the dialysable nitrogen was not absorbed on the Zeokarb and in this fraction there were at least three ninhydrin-positive substances, ethanolaminephosphoric acid, taurine and an unidentified acid-labile substance. On a two-dimensional chromatogram of collidine/lutidine and phenol/NH_3 the unknown substance appeared in approximately the position of β-alanine. A similar substance has also been identified in extracts of foetal lamb liver. The taurine was isolated in a crystalline form as follows.

The effluent was evaporated to dryness and most of the carbohydrate material was removed by repeated extraction with hot pyridine. Further purification was achieved by precipitation with phosphotungstic acid. The filtrate was then hydrolysed with 5N-HCl at 100°, evaporated to dryness and the HCl removed by passage through a column of Deacidite E. The effluent from this column was added to a Dowex 50 or Zeokarb 225 column. The only ninhydrin-positive substance present in the effluent from the latter column was taurine, which was obtained in crystalline form by evaporation of the effluent to a small volume and the addition of five times the volume of ethanol to the hot solution. A sample of taurine obtained by the above method from pig liver was chromatographically pure in phenol/NH_3, collidine/lutidine and amyl alcohol/pyridine and gave a satisfactory elementary analysis. A total of 22 mg. of taurine was isolated in the present case and it is calculated that 100 mg. could have been obtained from the whole pig liver weighing 1 kg.

The three ninhydrin-positive substances in the effluent from the Zeokarb 225 column could alternatively be precipitated as their mercury salts by the method of Neuberg & Kerb (1912). After decomposition of the salts by hydrogen sulphide the three substances could be further fractionated on a paper column with amyl alcohol/pyridine/water as solvent (Edman, 1945).

The use of the ion-exchange and paper columns was demonstrated.

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FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

The liberation of hydrogen sulphide by X-radiation from cysteine and glutathione. By W. M. Dale and J. V. Davies

An L-glutamic acid decarboxylase from barley. By H. Beevers

The component acids and glycerides of a horse mesenteric fat. By S. S. Gupta and T. P. Hilditch

Fish tropomyosin and fish nucleotropomyosin. By G. Hamoir

The effect of starvation of the pseudo-cholinesterase activity of the liver and serum of rats. By Marion F. Harrison and Lucy M. Brown

Studies in carotenogenesis. 1. General conditions governing β-carotene synthesis by the fungus *Phycomyces blakesleeanus* Burgeff. By G. A. Garton, T. W. Goodwin and W. Lijinsky

The effect of *cis*-vaccenic acid on respiration and growth of *Bacillus subtilis*. By H. Laser

Tricalcium phosphate as an adsorbent in the chromatography of proteins. By S. M. Swingle and A. Tiselius

The metabolism of nitrate and nitrite in the sheep. 1. The reduction of nitrate in the rumen of the sheep. By D. Lewis

The use of radioactive isotopes in immunological investigations. 5. Quantitative aspects of the antibody-antigen reactions. By T. E. Banks, G. E. Francis, W. Mulligan and A. Wormall

The biological action of substances related to thyroxine. 1. The effect of *n*-alkyl 3:5-diiodo-4-hydroxybenzoates on oxygen consumption in mice. By Mary M. Sheahan, J. H. Wilkinson and N. F. Maclagan

Metabolism of derivatives of toluene. 6. Tolunitriles, benzonitrile and some related compounds. By H. G. Bray, Z. Hybs and W. V. Thorpe

Biochemistry of locusts. 5. The green pigment of the haemolymph and integument of solitary locusts (*Locusta migratoria migratorioides*, R. & F., and *Schistocerca gregaria*, Forsk.). By T. W. Goodwin and S. Srisukh

Derivatives of adrenaline and noradrenaline in an extract of an adrenal medullary tumour. By T. B. B. Crawford

The preparation and properties of β-glucuronidase. 1. The fractionation of buffered water homogenates. By Lynda M. H. Kerr and G. A. Levvy

The micro-estimation of benzoic and hippuric acids in biological material. By F. Dickens and Joan Pearson

The aromatization of hydrogenated derivatives of benzoic acid in animal tissues. By C. T. Beer, F. Dickens and Joan Pearson

A note on the racemization of serine. By J. C. Crawhall and D. F. Elliott

Ultramicro determination of chloride. By R. Viswanathan

Improved manometric fluid. By H. A. Krebs

The lipid mobilized by anterior pituitary extract. By J. Campbell and C. C. Lucas

The effect of a high-sucrose diet on the calcium and phosphorus content of the enamel and dentine of rat incisors. By R. L. Hartles

Chemistry of tissues. 4. Methylation studies on hyaluronic acid. By M. A. G. Kaye and M. Stacey

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **291st Meeting** *of the Biochemical Society was held in the Department of Biochemistry, St Marys' Hospital Medical School, Paddington, London, W. 2, on Friday, 17 November, 1950, when the following papers were read:*

COMMUNICATIONS

The Effects of Steroid Hormones and of Gonadotrophins on Histaminase.

By R. Kapeller-Adler. (*Department of Pharmacology, University of Edinburgh*)

Serum histaminase is distinctly decreased in women suffering from severe pre-eclamptic toxaemia (Kapeller-Adler, 1944, 1949*b*, 1950).

Since it had been suggested that sex hormones might influence the activity of histaminase (Kapeller-Adler, 1944), the effects of various steroid hormones and gonadotrophins were investigated in the present work. The newly developed volumetric micromethod (Kapeller-Adler, 1950) was applied for the estimation of histaminase activity. Purified histaminase preparations made from hog kidneys (Kapeller-Adler, 1949*a*), as well as placental histaminase, were used as enzyme source.

A striking increase in histaminase activity was obtained with various oestrogens, for 2×10^{-6} M solutions of oestradiol and of oestrone and 2×10^{-5} M solutions of oestriol in phosphate buffer pH 7·2 increased the histaminase activity by about 50–80 %. The effects of these hormones increased proportionately with the amounts used. The effects of other steroid hormones are also being investigated, but a potentiation of histaminase activity has so far been obtained only with oestrogens.

A completely reversed effect on histaminase was obtained when preparations of chorionic gonadotrophins of different origins acted on both kidney and placental histaminase. The chorionic gonadotrophins used were International Standard preparations, Gonadex (prepared by Leo, Halsinborg), and preparations obtained from pregnancy urine by the author. 100 i.u. of these gonadotrophins decreased the histaminase activity by about 50 % on the average. A similar inhibitory effect on histaminase was observed with gonadotrophins from mares' serum. The effect of gonadotrophins on histaminase was found to be proportional to the amounts of the hormones used. The addition of chorionic gonadotrophins strongly decreased the potentiating effect of oestrogens on histaminase.

In view of the discrepancy in the behaviour of histaminase towards histamine and cadaverine (Kapeller-Adler, 1944, 1949*a*, 1950) the effects of steroid hormones and gonadotrophins on the histaminase-cadaverine reaction were also examined. An entirely opposite effect was obtained. Whereas the steroid hormones nearly completely abolished the action of histaminase on cadaverine, a strongly increased effect of the enzyme on cadaverine was observed with chorionic gonadotrophins and mares' serum gonadotrophin. This effect was found to be greatly reduced on addition of steroid hormones.

The results obtained in this work seem to suggest that the prosthetic group of histaminase, flavin-adenine dinucleotide (FAD), might be involved in these reactions. Previous observations (Kapeller-Adler, 1949*a*) showed that added FAD acts as activator of the histaminase-histamine reaction and as inhibitor of the histaminase-cadaverine reaction.

The significance of the results obtained in this work with regard to the behaviour of histaminase in normal and toxaemic pregnancy were discussed.

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The Valency of Antibodies.

By J. R. Marrack, H. Hoch and R. G. S. Johns. (*London Hospital Medical College, London, E.* 1)

The lattice theory of the precipitation and agglutination of antigens by antibodies is based on the assumption that antibodies are multivalent. The theory and the underlying assumption have been criticized by Hooker & Boyd (1942) and Boyd (1947).

Indirect evidence on the whole favours the lattice theory, but certain points are difficult to explain. Some direct evidence has been obtained from study of the soluble compounds formed by antibody with excess of antigen. From ultra-centrifuge studies

Pappenheimer, Lundgren & Williams (1940) concluded that diphtheria antitoxin combines with more than one, but not more than two, molecules of toxin; calculation from the data of one of the ultracentrifuge experiments of Heidelberger & Pedersen (1937) suggests that one molecule of antibody (rabbit) combined with only one molecule of the antigen, egg-albumin.

We have (1) formed a precipitate by adding an antigen (serum albumin) to the corresponding antiserum and redissolved this precipitate in excess of antigen; (2) prepared γ-globulin from antiserum and added this directly to excess of antigen. From the electrophoretic patterns of these solutions we have calculated the amount of antigen and antibody in the soluble complexes formed. In all cases the ratio of antigen to antibody molecules was between 1:1 and 2:1. It seems that some other factor besides bivalence of antibody molecules must be involved in the precipitation and aggregation of antigen-antibody complexes. This may be reduction of solubility of antibody molecules when several are combined with antigen. The failure of incomplete antibodies to aggregate or precipitate may be a matter of solubility rather than one of valency.

When excess antigen is added to the γ-globulin from antiserum the amount of γ-globulin that enters into the complex is considerably more than that precipitable by antigen.

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The Transamination and Oxidation of L-Tyrosine by Rat Liver with the Formation of Acetoacetate. By M. Le May-Knox (*Department of Radiotherapeutics, University of Cambridge*) and W. E. Knox* (*The Molteno Institute, University of Cambridge*)

Tyrosine oxidation has been obtained in concentrated suspensions of liver (Sealock & Goodland, 1949), and the activity of these preparations can be increased somewhat by ascorbic acid (Painter & Silva, 1950). The reaction catalysed has not been determined nor have either of the alternative pathways of tyrosine metabolism suggested by intact animal experiments been confirmed (Pitt-Rivers, 1950).

Upon testing whether tyrosine oxidation in liver, like the oxidation of tryptophan (Knox & Mehler, 1950), might involve a peroxidase requiring enzymically generated H_2O_2, it was found that the addition of D-amino-acid oxidase plus DL-alanine to the rat-liver homogenate tripled the rate of L-tyrosine oxidation. However, this increase was due, not to the H_2O_2 generated, since added notatin plus glucose is without effect, but to the pyruvate formed by the oxidation of alanine. α-Ketoglutarate addition is even more effective than pyruvate in increasing the rate of tyrosine oxidation.

A transamination reaction, which limits the overall oxidation of tyrosine is suggested by this effect of pyruvate and α-ketoglutarate. This has been confirmed by finding stoicheiometric amounts of L-glutamate formed from the added α-ketoglutarate during the tyrosine oxidation (the specific L-glutamic acid decarboxylase of *Clostridium welchii* was provided by Miss Van Haltern). The initial reaction is a tyrosine-α-ketoglutarate transamination forming *p*-hydroxyphenylpyruvate, since little tyrosine disappears in the absence of α-ketoglutarate. Such a tyrosine-α-ketoglutarate transaminase has previously been demonstrated in liver by Mr E. V. Rowsell (personal communication, 1950).

When this transamination reaction is no longer limiting, an analysis of the extent of the oxidation of tyrosine and of its intermediate steps becomes possible. The oxygen uptake approximates the theoretical value of 4 atoms per molecule of tyrosine oxidized, with formation of one molecule of acetoacetate determined by aniline citrate. As expected from the initial occurrence of transamination, *p*-hydroxyphenylpyruvate (a gift from Mr E. V. Rowsell) is oxidized without need of α-ketoglutarate at nearly the same rate as is tyrosine with α-ketoglutarate.

Ascorbic acid is even more effective in freshly dialysed rat-liver preparations than reported for liver homogenates from scorbutic guinea pigs. About 5 μmol. of additional tyrosine is oxidized in the presence of 0·2 μmol. of ascorbic acid. Neither cysteine nor slowly generated H_2O_2 can replace it.

* Special Research Fellow of the U.S. Public Health Service.

Table 1. *The oxidation of* L-*tyrosine to acetoacetate with the formation of glutamate*

(μmol. of reaction after 2 hr. incubation of 0·5 g. rat liver with 13 μmol. L-tyrosine, plus 40 μmol. α-ketoglutarate and/or 0·8 μmol. ascorbic acid, pH 7·2.)

Liver preparation		Additions: None	Ascorbic acid	α-Keto-glutarate	Ascorbic acid and α-keto-glutarate
Homogenate	L-Tyrosine	−0·2	−0·2	−4·8	−10·1
	Acetoacetate	—	—	+4·1	+8·7
Supernatant	L-Tyrosine	−0·1	−0·3	−4·9	−10·1
	Acetoacetate	—	—	+5·0	+10·3
Dialysed supernatant	L-Tyrosine	—	—	−3·0	−9·5
	Acetoacetate	—	—	+2·9	+9·3
	L-Glutamate	—	—	+4·8	+8·4

Table 2. *Ascorbic acid requirement for tyrosine oxidation by a dialysed soluble preparation of rat liver* (μmol. *of reaction in* 1 *hr.*)

	Additions: None	Ascorbic acid: $0·3 \times 10^{-4}$ M	Ascorbic acid: $1·6 \times 10^{-4}$ M	Ascorbic acid: $8·0 \times 10^{-4}$ M	Cysteine 0·001 M	Glucose-notatin
Tyrosine	−2·3	−2·3	−7·5	−7·7	−2·0	−0·8
Acetoacetate	+2·6	+2·4	+6·5	+6·0	—	—

The effect of ascorbic acid is demonstrable with *p*-hydroxyphenylpyruvate as well as with tyrosine as substrate. During the slow reaction of tyrosine in the absence of ascorbic acid, in analogy with the metabolism of the scorbutic guinea pigs (Painter & Silva, 1947), some *p*-hydroxyphenylpyruvate accumulates along with glutamate.

p-Hydroxyphenylpyruvate is apparently oxidized through homogentisic acid, since the soluble tyrosine oxidizing system of liver is also able to oxidize homogentisic acid to acetoacetate (cf. Ravdin & Crandall, 1950). This reaction, assayed with samples of homogentisic acid presented by Dr A. Neuberger and Dr Karl Meyer, occurs at 100 times the rate of the overall tyrosine oxidation and is not influenced by ascorbic acid.

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An Activator of Hexokinase in Animal Tissues. By H. Weil-Malherbe. (*Runwell Hospital, Wickford, Essex*)

In a previous communication (Weil-Malherbe, 1948) the activation of brain hexokinase by erythrocyte lysates was reported. A similar or identical activator is also present in muscle. The activator is a specific protein which, though devoid of hexokinase activity itself, may cause a two- to four-fold increase of the hexokinase activity of brain, muscle or yeast preparations. The effect is not associated with any of the following enzymes: adenosinetriphosphatase (ATPase), phosphohexokinase, myokinase, adenylic deaminase, aldolase or triose phosphate dehydrogenase. The activation is specific for the phosphorylation of glucose and fructose and does not affect the phosphorylation of fructose-6-phosphate.

The increase in the rate of glucose phosphorylation is the only observable effect of the activating factor; the ratios of glucose disappearance to the utilization of acid-labile phosphate groups, the formation of hexosediphosphate and the appearance of acid are not significantly changed in the presence of the activator. Neither is there any increase in the relative utilization of adenosine diphosphate.

Brain hexokinase is strongly inhibited by hexose-monophosphates. This inhibition is proportionally

the same in the presence and in the absence of the activator.

A characteristic feature of the activator is its relatively great heat stability. The factor from muscle can be heated to 80° for 5 min. at pH 7·5 without great loss of activity, and the factor from erythrocytes will even stand a temperature of 100° for a short time. In contrast to myokinase, however, activity is lost if the factor is heated in acid solution.

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The Catalytic Effect of Molybdate on the Hydrolysis of Phosphate Bonds. By H. Weil-Malherbe. (*Runwell Hospital, Wickford, Essex*)

It is well known that molybdate catalyses the hydrolysis of creatine phosphate and acetyl phosphate. The demonstration of this effect with the aid of methods of phosphate estimation of the Fiske and Subbarow type involves a preliminary separation of organic and inorganic phosphate. In the extraction methods of phosphate estimation (Berenblum & Chain, 1938; Martin & Doty, 1949) the time in which organic and inorganic phosphates are simultaneously in contact with molybdate can be reduced to a few seconds. In the case of an extremely labile ester, such as acetyl phosphate, the separation method must be employed, but with most of the other phosphate esters, including creatine phosphate, the molybdate effect can be investigated by extraction methods. Using a modification of the method of Martin & Doty (1949) a molybdate effect could be demonstrated with every ester investigated though it varied quantitatively. In Table 1 the values of some monomolecular reaction constants of the hydrolysis in presence and in absence of molybdate and their ratios are shown.

Of particular interest is the fact that the molybdate effect often has a well pronounced pH optimum which varies from ester to ester, e.g. for adenosinetriphosphate (ATP) the optimum pH is about 4. This may cause errors in phosphate estimations according to Lowry & Lopez (1946).

The hydrolysis curve of ATP is formed by the superimposition of two monomolecular reaction curves, the hydrolysis constant of the terminal phosphate group being over 10 times greater than that of the intermediate phosphate group.

Table 1. *The effect of* 1 % *ammonium molybdate on the hydrolysis of some phosphoric esters*

k_m, monomolecular reaction constant for hydrolysis in presence of molybdate.
k_c, monomolecular reaction constant for hydrolysis in absence of molybdate (control).

Ester	pH	Temp. (°)	k_m	k_c	k_m/k_c
Acetyl phosphate	4·0	0	0·226	0·00112	202
ATP (terminal group)	4·1	70	0·078	0·00050	156
Creatine phosphate	2·5	0	0·0543	0·00040	135
Glucose-1-phosphate	3·3	70	0·00197	0·000312	6·3
Fructose-6-phosphate	5·8	80	0·00417	0·00082	5·1
Sodium pyrophosphate	2·2	80	0·0245	0·0053	4·6
Phospho-enol-pyruvate	4·0	80	0·055	0·0125	4·4
α-Glycerophosphate	6·3	80	0·000364	0·000150	2·4
Glucose-6-phosphate	6·3	80	0·000306	0·000155	2·0

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Cholinesterase Inhibition by Parathion (*p*-Nitrophenyldiethylthiophosphate). By W. M. Diggle and J. C. Gage. (*Imperial Chemical Industries Ltd., Industrial Hygiene Research Laboratory, Welwyn, Herts*)

We have investigated the cholinesterase inhibition of several samples of parathion from various sources and of certain analogous compounds. A rat-brain homogenate has been used as a source of specific cholinesterase with $1{\cdot}2 \times 10^{-4}$ M-acetylcholine chloride as substrate; the hydrolysis has been followed by the chemical method of Hestrin (1949). At this substrate concentration the hydrolysis at 37° is first order until it has proceeded about half-way to completion. The inhibitors were incubated with the enzyme preparation for 30 min. at 37°. Certain impurities present in the samples of parathion have been estimated by a chemical method of analysis which will be described in detail elsewhere.

We find that the power of the parathion samples to inhibit cholinesterase, determined by the reciprocal of the concentration required to produce a 50% inhibition, is approximately proportional to their content of the *S*-ethyl isomer of parathion. The inhibitory power of a highly purified sample of parathion containing only a small amount of this isomer is low, about 20 μg./ml. being required to achieve a 50% inhibition; that of the technical grade of parathion may be considerably reduced after a sample has been shaken with dilute sodium hydroxide, which attacks the *S*-ethyl isomer. This isomer and the oxygen analogue of parathion, E 600, are potent inhibitors, of the order of 10^4 and 10^5 times as active respectively as the purest sample of parathion. The *S*-phenyl isomer also shows an inhibition greater than can be accounted for by its content of *S*-ethyl isomer. Measurements of the brain cholinesterase of rats which have been dosed intraperitoneally with parathion suggest that parathion has an *in vivo* inhibitory action which cannot be ascribed solely to the presence of impurities.

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Effect of Vitamin B Deficiency on Nucleic Acid Phosphorus Content of Rat Bone Marrow. By C. Lutwak-Mann. (*Biochemical Laboratory, University of Cambridge*)

In the course of studies on the metabolism of bone marrow (Lutwak-Mann, 1947*a*, *b*, 1949; Lutwak-Mann & Gunz, 1949), the deoxyribonucleic acid (DN) and ribonucleic acid (RN) P content was analysed in the bone marrow of rats maintained on

Table 1. *Effect of diet on the nucleic acid phosphorus content of rat bone marrow*

	Nucleic acid (mg. P/100 g.)		
	DN	RN	Total
Full synthetic diet	149	87	236
	173	82	255
	164	85	249
	163	92	255
	166	75	241
	169	79	248
	178	94	272
	180	95	275
	159	88	247
	160	78	238
Full synthetic diet + 200 μg. aneurin daily	164	86	250
	169	79	248
Full synthetic diet restricted by pair-feeding	147	67	214
	134	74	208
Pair-feeding + 100 μg. aneurin daily	141	74	215
	137	73	210
Pair-feeding + 10 μg. vitamin B_{12} daily	135	67	202

Table 1 (*cont.*)

	Nucleic acid (mg. P/100 g.)		
	DN	RN	Total
B-deficient diet	69	42	111
	98	63	161
	105	59	164
	95	54	149
	112	59	171
	158	68	226
	110	60	170
	103	57	160
	105	61	166
B-deficient + 100 μg. aneurin daily	125	81	206
	137	71	208
	149	93	242
	152	73	225
B-deficient + 0·4 ml. 'B-complex' daily	156	94	250
	138	83	221
B-deficient + 10 μg. vitamin B_{12} daily	153	58	211
	122	55	177
	93	95	188
B-deficient + 0·5 mg. folic acid daily	114	61	175
	88	46	134
B_1-deficient diet	64	34	78
	85	49	134
	79	61	140
	113	67	180
B_1-deficient + 100 μg. aneurin daily	100	55	155
	146	80	226

a vitamin B- or B_1-deficient diet, and compared with controls kept on a full synthetic diet consumed either *ad lib.* or restricted by pair-feeding with the deficient rats. Laboratory stock hooded male rats 3–4 months old were used. The deficiency period lasted for 4 weeks; then for another 2 weeks some of the deficient animals and some of the control animals received parenterally the vitamins indicated in Table 1.

It can be seen that quantitative restriction of non-deficient food due to pair-feeding, caused a fall in nucleic acid P, not affected by treatment with aneurin or vitamin B_{12} (Cytamen, Glaxo). Excess aneurin in normal rats was without effect.

Rats deficient in group B vitamins or B_1 only, showed considerably lower values than controls. Distinct improvement, both in the general condition of the rats as well as the nucleic acid P of the bone marrow, resulted from treatment with 'B complex' mixture (kindly supplied by Dr E. Kodicek, Nutrition Laboratory, Cambridge) or aneurin, on unrestricted food intake, but not with B_{12} or folic acid (Folvite, Lederle).

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Effect of Malnutrition on the Secretory Function of Male Accessory Glands of Reproduction.

By C. LUTWAK-MANN and T. MANN. (*Biochemical Laboratory and the Molteno Institute, University of Cambridge*)

It was shown previously (Lutwak-Mann & Mann, 1950) that the secretion of fructose and citric acid in the male accessory glands is severely curtailed in vitamin B-deficient rats, but can be fully restored with testosterone propionate or chorionic gonadotrophin, irrespective of food intake. We now find that vitamin B_1 deficiency causes a reduction of the secretory function in the same manner as general B deficiency. Furthermore, the secretory process is also seriously affected by quantitative restriction of the full diet to the level of the vitamin B-deficient animals (pair-feeding); this is seen in Table 1, which gives values for the citric acid and fructose content based on six rats per group.

With regard to the influence of vitamins upon the secretion of the accessory glands, it was found that parenteral administration of aneurin had no effect in rats on a full diet, and only a slight one in animals whose food intake was limited by pair-feeding. In rats maintained on vitamin B- or B_1-deficient diets, distinct improvement followed treatment with aneurin, but only if food intake was not restricted through pair-feeding. Similar results were obtained with 'B complex' mixture. On the other hand, injections of B_{12} or folic acid led to a lowered level of fructose and citric acid in the accessory organs.

Table 1. *Effect of diet on the secretory function of male accessory glands of reproduction*

Diet	Fructose	Citric acid	
	Coagulating gland (μg./rat)	Seminal vesicles (μg./rat)	Ventral prostate (μg./rat)
Full synthetic	328–649	456–675	424–681
B deficient	42–156	64–135	136–360
Full synthetic restricted by pair-feeding	250–315	166–170	370–434

REFERENCE

Lutwak-Mann, C. & Mann, T. (1950). *Nature, Lond.*, **165**, 556.

The Metabolism of Nitrobenzene and Mononitrophenols in Rabbits. By D. ROBINSON, J. N. SMITH and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Hitherto, the only proved metabolite of nitrobenzene is *p*-aminophenol (cf. Meyer, 1905), and despite its importance we know very little about the fate of nitrobenzene in the body.

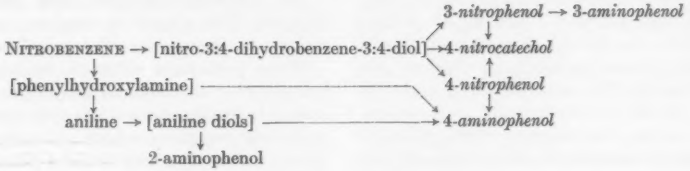
As a preliminary to the study of nitrobenzene, the metabolism of the three mononitrophenols was studied. These three phenols are largely excreted as glucuronides (isolated as triacetyl methyl esters) for 80% or more of the nitro group is excreted in this form (see Table 1). There is little reduction to aminophenols. Reduction is least with *o*-nitrophenol and greatest with *p*-nitrophenol. The nitrophenols are also very slightly oxidized (<0·1%), and by paper chromatography, combined with spectroscopy, we have found that the *ortho* compound gives rise to nitroquinol (approx. 0·03%), the *meta* compound gives 4-nitrocatechol (0·03%) and a trace of nitroquinol, and the *para* compound gives 4-nitrocatechol (<0·1%).

Table 1. *The metabolism of nitrophenols*

Nitrophenol fed	NO_2 group excreted (%)	Reduction to aminophenol (%)	Total excretion (%)
ortho	82	2·5	84·5
meta	75	9·5	84·5
para	87	14	101

Nitrobenzene, however, is completely metabolized and extensively reduced in the rabbit. Preliminary determination suggests that 40% is excreted as aminophenols and 20% as nitrophenols. From the nitrophenol fraction of nitrobenzene urine we have isolated, by partition chromatography on silica, *m*- and *p*-nitrophenols and 4-nitrocatechol (characterized as benzoates). The *m*- and *p*-nitrophenols occur in roughly equal amounts. *p*-Aminophenol has also been isolated in quantity. The presence of *m*-aminophenol has been proved and we suspect also the presence of *o*-aminophenol and aniline in the urine. Examination of the urine for a nitrobenzene-diol (cf. chloro-3:4-dihydrobenzene-3:4-diol; Smith, Spencer & Williams, 1950) has so far yielded no positive result. The following scheme is suggested to explain our results.

Compounds in brackets are hypothetical, those in italics have been proved to be formed and formation of the others is suspected.



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Metabolism of Benzene. Excretion of Phenylsulphuric acid after Administration of Phenylmercapturic Acid and Phenylcysteine. By D. V. PARKE and R. T. WILLIAMS. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Suggestions have been made that the ethereal sulphates formed from aromatic hydrocarbons in the body are derived from a sulphur-containing intermediate, and phenylmercapturic acid has been suggested as such an intermediate. We have therefore studied the fate of phenylmercapturic acid, a metabolite of benzene, and of *S*-phenyl-L-cysteine in rabbits. Table 1 gives a summary of our results.

Both phenylmercapturic acid and phenylcysteine cause the excretion of increased amounts of ethereal sulphate which was shown to be that of phenol by the isolation of the latter (as the toluene-*p*-sulphonate) from the hydrolysed urine. The extra ethereal sulphate appeared on the second day after feeding the mercapturic acid, but usually on the first day after phenylcysteine.

Table 1. *The metabolism of phenylmercapturic acid and phenylcysteine*

Compound administered	Percentage of dose found in urine as: Ethereal sulphate	Mercapturic acid	Phenylcysteine	Thiophenol	Diphenyl disulphide
Phenylmercapturic acid	10	45	14	0·4	—
Phenylcysteine	8	24	24	4	5·2

Phenylmercapturic acid also underwent some deacetylation, and not more than 50% was excreted unchanged whether fed or injected. About 25% of the phenylcysteine administered was acetylated.

There was an appreciable excretion of thiophenol after phenylcysteine administration, although only small amounts were found after giving phenylmercapturic acid. The diphenyl disulphide in phenylcysteine urine is probably produced by aerial oxidation of thiophenol so that nearly 10% of phenylcysteine is converted to thiophenol. One probable path of metabolism of phenylmercapturic acid is:

$$C_6H_5SCH_2CH(NHAc)COOH \rightleftarrows C_6H_5SCH_2CH(NH_2)COOH \rightarrow C_6H_5SH.$$

It seems likely that the phenol of the phenylsulphuric acid is derived from phenylmercapturic acid, but whether or not the sulphate is derived from the same source cannot be proved in this type of experiment.

Spectrophotometric Determination of Phenolsulphatase (Arylsulphatase). By D. Robinson, B. Spencer and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

In the past, determinations of arylsulphatase activity have involved incubation for prolonged periods varying from 3 hr. to several days, usually about 10 hr. Using the present methods, incubation time can be reduced to 1 hr. The principle of the method is to determine, spectrophotometrically as its anion, the phenol set free by the enzyme, selecting for the purpose a phenol whose anion absorbs maximally at a wave length where the absorption of its ethereal sulphate is negligible. Suitable phenols were found in *p*-chlorophenol, *p*-nitrophenol and *p*-hydroxybenzaldehyde, and the relevant data are given in Table 1.

The ethereal sulphates used are readily synthesized. The source of enzyme used was takadiastase, a preparation of *Aspergillus oryzae*.

When potassium chlorophenylsulphate is used, protein must be removed with ethanol from the incubated solution, since it has appreciable absorption at the wavelengths used for estimating the *p*-chlorophenol anion (cf. estimation of β-glucuronidase using *p*-chlorophenylglucuronide; Spencer & Williams, 1950), but with the *p*-nitro- and *p*-aldehydo-phenylsulphates this may not be necessary unless large amounts of protein are present.

Table 1. *Molecular extinction coefficients* (ε) *of phenols and their ethereal sulphates at certain wavelengths*

Compound	ε in 0·1 N-NaOH at: 245 mμ.	298 mμ.	330 mμ.	402 mμ.
p-Chlorophenol	11,650	2,600	—	—
K *p*-Chlorophenylsulphate	8·5	0·73	—	—
p-Hydroxybenzaldehyde	—	—	27,500	—
K *p*-Aldehydophenylsulphate	—	—	140	—
p-Nitrophenol	—	—	—	17,000
K *p*-Nitrophenylsulphate	—	—	—	16

Table 2. *Sulphatase action of takadiastase*

Ethereal sulphate substrate	Optimum pH	$K_m \times 10^4$	Phenol released*/hr.: μg.	μmol.
p-Chlorophenylsulphate	6·15	9·7	12·9†	0·100
p-Nitrophenylsulphate	6·1	1·2	10·3	0·074
p-Aldehydophenylsulphate	5·8	2·1	7·7	0·063

* Arbitrary conditions. † 2 hr. incubation period.

The action of takadiastase on these substrates was studied at 37° in 0·5M-acetate buffer, the incubation periods being 2 or 3 hr. for the chloro-compound, 1 or 2 hr. for the nitro-compound and 1 hr. for the aldehydo compound. The Michaelis constants K_m and pH optima are given in Table 2.

Whilst all three substrates can be used for sulphatase estimation, the *p*-nitro- and *p*-aldehydo-compounds are better than the *p*-chloro-compound, because the maxima for the first two are higher and are at longer wavelengths than those of the *p*-chloro-compound. With the Unicam spectrophotometer the convenient amounts of the phenols for measurement are, *p*-aldehydo 2–9 μg., *p*-nitro 3–14 μg. and *p*-chloro 10–50 μg.

In agreement with earlier workers, we found that the enzyme was inhibited by phosphate and sulphite, but not by chloride and sulphate ions. These and other results were discussed.

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Glucuronide Synthesis in Liver Homogenates. By G. J. Dutton and I. D. E. Storey. (*Departments of Biochemistry and of Surgery, University of Edinburgh*)

Although the synthesis of glucuronides by liver slices may be readily demonstrated, various workers have been unable to show such a reaction in broken liver preparations. In the present study, mouse liver was homogenized in alkaline isotonic KCl, and incubations were carried out in a medium containing phosphate buffer and Mg^{++}, but no Na^{+}, and in presence of *o*-aminophenol and ascorbic acid (Levvy & Storey, 1949). A synthesis of *o*-aminophenylglucuronide could not, however, be detected under these conditions unless a boiled extract of liver was also added.

The activity of such extracts is rapidly lost when they are stored at 0°, but an active powder may be prepared by extracting finely minced rabbit liver with trichloroacetic acid in the cold. Glycogen is precipitated from the acid solution by ethanol, and the clear supernatant liquid after centrifugation is poured into excess acetone. When 3·6 mg. of the most active preparation so far obtained was added to a homogenate containing 50 mg. (wet weight) liver in 3 ml. medium, 11·7 μg. *o*-aminophenol were conjugated as glucuronide. This rate of synthesis is of the same order as that found with liver slices (Storey, 1950). The powder loses activity slowly even when stored *in vacuo* over $CaCl_2$ in the refrigerator, and rapidly when warmed in acid solution.

No synthesis was observed when the powder was replaced by any of the following added singly: adenylic acid, adenosinetriphosphate (ATP), cozymase, flavin-adenine dinucleotide, aneurin, riboflavin, pyridoxin, nicotinamide, pantothenate, folic acid, coenzyme *A*, cytochrome *c*, boiled extracts of yeast and wheat germ, D-ribose, D-xylose, glucose-1-phosphate, acetate, pyruvate, succinate, fumarate, L-glutamate or Mn^{++}. Various combinations of dicarboxylic acids, adenylic acid, ATP and cytochrome *c* likewise failed to reproduce the effect of the extracts.

In phosphate or veronal buffers the system shows a broad pH maximum in the region pH 7·6–7·8. Mg^{++} ions exert a stimulating effect on the synthesis, but saccharate (0·001 or 0·0001M), added to inhibit β-glucuronidase activity (Karunairatnam & Levvy, 1949), did not increase *o*-aminophenylglucuronide formation.

We are indebted to Mr W. E. Balfour, Department of Physiology, University of Edinburgh, for samples of flavin-adenine dinucleotide and an active coenzyme *A* preparation, and to Dr A. B. Roy for glucose-1-phosphate.

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The Estimation of Sodium Gentisate in Plasma or Serum. By B. W. Meade and M. J. H. Smith, (*Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London, S.E.* 5)

Sodium gentisate is claimed to be as active therapeutically as sodium salicylate in the treatment of rheumatic fever (Meyer & Ragan, 1948) and has the advantage that it produces few, if any, toxic effects. Camelin, Accoyer, Pellerat, Lafuma & Coirault (1949) stated that the concentration of the drug in plasma could be measured by utilizing the blue colour given with ferric chloride and reported levels

of up to 10 mg. of sodium gentisate/100 ml. when the drug was administered orally. However, Meyer & Ragan (1948) failed to detect any gentisate in the blood of patients receiving similar doses. Using the method to be described, samples of plasma from patients receiving 12 g. of sodium gentisate/day gave values ranging from 4 to 11 mg./100 ml.; samples from patients not receiving gentisate therapy gave negligible blank values.

The method is based on the observation that gentisic acid is completely extracted from acidified plasma or serum by ethyl acetate and quantitatively removed from the organic solvent with 1% (w/v) sodium bicarbonate solution. The gentisic acid may then be estimated by means of its reaction with an aqueous solution of the Folin-Ciocalteu phenol reagent (Smith, 1950). A blue colour having an absorption maximum at 660 mμ. develops on the addition of sodium hydroxide. The optical density of the coloured solution was found to be proportional to the concentration of gentisate ion up to 12 mg./100 ml. and the method gave a recovery of 96% from plasma or serum. A calibration curve may be constructed from dilutions of sodium gentisate in distilled water.

The method of estimation is as follows: 0·5 ml. of 6N-hydrochloric acid is added to 2 ml. of plasma or serum followed by 10 ml. of ethyl acetate. The mixture is shaken for 2 min. and centrifuged for 5 min.; 5 ml. of the ethyl acetate layer are removed and added to 5 ml. of 1% (w/v) sodium bicarbonate solution. This mixture is shaken for 2 min. and centrifuged for 5 min.; 4 ml. of the bicarbonate layer are removed and 1 ml. of Folin-Ciocalteu reagent (diluted 1 to 3 with distilled water) is added and mixed. After the addition of 1 ml. of 1·5N-sodium hydroxide, the solution is allowed to stand for 5 min. and the optical density measured against distilled water in a photo-electric absorptiometer using a 1 cm. cell and an Ilford spectrum red filter no. 608 transmitting maximally above 660 mμ.

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The Evolution of Dimethyl Sulphide from Bracken, 'Horse's Tail' and Other Plants on Treatment with Sodium Hydroxide. By R. Bywood, F. Challenger, D. Leaver and Margaret I. Whitaker. (*Department of Organic Chemistry, The University, Leeds*)

The evolution of dimethyl sulphide from *Polysiphonia fastigiata* on exposure to air or treatment with sodium hydroxide (Haas, 1935), the isolation of dimethyl-β-carboxyethylsulphonium chloride from this alga by Challenger & Simpson (1948) and its recognition as a methyl donor (Maw & du Vigneaud, 1948; Dubnoff & Borsook 1948) suggested a search for other plants exhibiting similar behaviour. Lupin seeds, unroasted coffee beans and seeds of Indian mustard (*Brassica juncea*) with sodium hydroxide gave no dimethyl sulphide. The detection of dimethyl sulphone in the Equisetae ('Horse's Tail') by Karrer & Eugster (1949) suggested to us that this might arise from a sulphonium compound through dimethyl sulphide followed by oxidation, although decomposition of a compound such as $CH_3SO_2CH_2COOH$ might possibly be responsible.

With hot sodium hydroxide in an air stream the intact plants *Equisetum telmateia* (*maximum*), *E. limosum* and the spore-bearing cones of *E. palustre* yield small quantities of dimethyl sulphide which was characterized as the mercurichloride after passage through hydrochloric acid (to remove bases), mercuric cyanide (no precipitate, therefore no mercaptan) and finally mercuric chloride. The sulphide did not arise from dimethyl sulphone by reduction as on heating this with sodium hydroxide, glucose and filter paper to simulate the experimental conditions no dimethyl sulphide was formed. The reduction of the very stable sulphone under such conditions was hardly to be expected.

Evans, Jones & Evans (1950) and Roberts, Evans & Evans (1950) found that extracts of *E. arvense* and of bracken (*Pteris aquilina*) cause a B_1 avitaminosis in horses and rats. Bracken poisoning in cows appears to be much more complex. This suggested an examination of the alkaline decomposition of bracken and it was found that this plant, especially the young shoots, readily evolved dimethyl sulphide, characterized by the m.p. and mixed m.p. of the mercurichloride, the sulphilimine, $(CH_3)_2S{\rightarrow}N.SO_2C_6H_4CH_3$, and of benzyldimethylsulphonium picrate. The fern *Athyrium filix-foemina* also yields small quantities of the same sulphide with hot alkali. The quantity of mercurichloride was only sufficient for recognition of the odour of the sulphide evolved on treatment with alkali.

Prof. P. Karrer agreed that we should examine

these plants for the presence of sulphonium compounds and our experiments are in progress.

One of us (M.I.W.), during a study of the sulphur compounds of asparagus (other than the 1:3-dimercaptopropane-2-carboxylic acid isolated by Jansen, 1948) in progress since 1947, finds that an evaporated acetone extract with boiling alkali gives traces of dimethyl sulphide characterized as the mercurichloride.

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Liver Glutathione and Ascorbic Acid in Dietetic Liver Necrosis of Rats. By O. LINDAN and ELIZABETH WORK. (*University College Hospital Medical School, London, W.C.* 1)

Dietetic liver necrosis in rats is produced by the double deficiency of sulphur-containing amino-acids and tocopherol. Diets containing only 7 % protein, all of which is provided by dried baker's yeast (necrogenic yeast diet), produce hepatic necrosis and death of tocopherol-depleted weanling rats in about 28 days (Lindan & Himsworth, 1950; Lindan & Work, 1951). Supplements of either tocopherol, cystine or methionine will protect the animal.

As glutathione is the only sulphur-containing component of the liver known to vary with changes in the dietary protein sulphur (Leaf & Neuberger, 1947), its level during necrosis was investigated. Glutathione and ascorbic acid concentrations were examined (titration method of Leaf & Neuberger) in liver, blood and spleen of rats fed the necrogenic yeast diet, with or without additional sulphur-containing amino-acids or tocopherol. The unsupplemented diet led to no change in non-protein nitrogen, but a fall occurred in the reduced and total glutathione and ascorbic acid of the liver within 12 days, after which there was no further change until necrosis developed. Necrotic livers, removed from moribund rats, showed even lower reduced and total glutathione levels and no ascorbic acid

Table 1. *Effect of necrogenic yeast diet and of liver necrosis on glutathione, extractable nitrogen and ascorbic acid contents of liver, spleen and blood*

	Stock diet (liver normal)	Necrogenic yeast diet	
		Liver not necrotic	Liver necrotic
Liver weight (g./100 g. body wt.)	4·4	5·0	8·9
Liver glutathione (mg./100 g. liver):			
reduced	217	64	25
total (reduced + oxidized)	234	84	49
Liver glutathione, $\frac{\text{oxidized}}{\text{reduced}} \times 100$	8	31	96
Liver reduced glutathione (mg./100 g. body wt.)	9·6	3·2	2·2
Liver extractable nitrogen (mg./100 g. liver)	186	170	95
$\frac{\text{Total liver glutathione N} \times 100}{\text{Extractable N}}$	22	6·7	7·2
Liver ascorbic acid (mg./100 g.)	48	26	0
Spleen ascorbic acid (mg./100 g.)	40	27	33
Spleen reduced glutathione (mg./100 g.)	113	67	101
Blood reduced glutathione (mg./100 g.)	22	13	22

(Table 1). The amount of oxidized glutathione, however, increased slightly after 14 days, but was subsequently raised in necrosis, thus resulting in increased ratios of oxidized to reduced glutathione. The fall in total glutathione from the pre-necrotic to the necrotic levels was paralleled by a similar fall in extractable nitrogen. These changes cannot be explained simply by dilution through the swelling of the liver in necrosis.

Reduced glutathione in blood and spleen dropped after 14 days on the diet, but became normal in necrotic rats. Ascorbic acid in spleen did not disappear in such rats (ascorbic acid in blood was too low to be estimated by our method).

Tocopherol supplements had no effect on the pre-necrotic fall in reduced glutathione, although they did prevent necrosis and the terminal fall; a daily supplement of as little as 0·1 % cystine and 0·05 % methionine kept the glutathione normal.

Changes in liver glutathione, non-protein nitrogen and ascorbic acid similar to those occurring in dietetic necrosis were produced in 18 hr. either by lightly ligaturing one lobe of the liver so that stasis and subsequent necrosis developed, or by excising one lobe and leaving it in the abdominal cavity. Sterile incubation of excised liver outside the body produced completely different changes (Table 2).

It is not yet possible to assess the significance of these changes in dead or dying liver in relation to those found in dietetic liver necrosis.

Table 2. *Effect of death of liver on glutathione, extractable nitrogen and ascorbic acid contents*

(Values expressed as mg./100 g. liver.)

Liver lobe	Glutathione		Extractable nitrogen	Ascorbic acid
	Reduced	Total		
Ligatured	30	40	82	0
Control	270	280	230	34
Excised and left in abdomen	12	54	77	0
Control	273	—	—	24
Excised and incubated at 37°	145*	280*	836	27
Control	231	244	242	37

* Value unreliable, as free cystine and cysteine were shown by paper chromatography to exceed glutathione.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **292nd Meeting** *of the Biochemical Society was held in the Department of Biochemistry and Chemistry, The Medical College of St Bartholomew's Hospital, Charterhouse Square, London, E.C. 1, on Friday, 15 December, 1950, when the following papers were read:*

COMMUNICATIONS

Some Observations on the Chloroform-soluble Conjugated Adrenocortical Steroids in Human Urine.

By J. Y. F. Paterson and G. F. Marrian. (*Department of Biochemistry, University of Edinburgh*)

It has previously been shown (Paterson, Cox & Marrian, 1950) that human urine contains two different types of conjugated formaldehydogenic compounds (assumed to be conjugated steroids derived from the adrenal cortex), one of which is readily extracted from acidified urine by chloroform, while the other is not. In this previous communication, evidence was presented which suggested that the free steroid obtained from the former type of conjugate is much less stable at pH 1 and 25° than that obtained from the latter type. It is now known, however (see following communication), that the latter type of conjugate yields on hydrolysis both acid-labile and acid-stable free formaldehydogenic steroids. Accordingly, a difference in the stability to acid of the free steroids is no longer considered to be a satisfactory basis for the classification of the conjugated compounds.

In the present communication a further study of the properties of the chloroform-extractable type of conjugated formaldehydogenic steroid is reported. Concentrates of the conjugate were prepared by the following method: immediately after acidification to pH 1 the urine was extracted with chloroform, and the conjugate was obtained from this extract by extraction with aqueous $NaHCO_3$.

At pH 3–4 and 25° the conjugate is hydrolysed to the free formaldehydogenic compound in 1–3 hr. The free compound is stable at this pH for at least 6 hr. No hydrolysis of the conjugate could be obtained at pH 5 with active preparations of ox-spleen β-glucuronidase.

REFERENCE

Paterson, J. Y. F., Cox, R. I. & Marrian, G. F. (1950). *Biochem. J.* **46**, xxix.

The Hydrolysis of the Chloroform-insoluble Conjugated Adrenocortical Steroids in Human Urine.

By R. I. Cox and G. F. Marrian. (*Department of Biochemistry, University of Edinburgh*)

Concentrates containing part of the conjugated formaldehydogenic steroids not extractable from acidified urine by chloroform (see preceding communication) were prepared in the following manner: after extraction of urine with chloroform to remove free steroids and chloroform-soluble conjugates, the acidified urine was extracted with *n*-butanol. The conjugated formaldehydogenic steroids were then extracted from the *n*-butanol by shaking with aqueous NaOH.

Incubation of such extracts at 37° and pH 5 with crude preparations of ox-spleen β-glucuronidase yielded free formaldehydogenic steroid in amounts about ten times greater than could be obtained by acid hydrolysis for 24 hr. at pH 1 and 37°.

Greatly increased yields of formaldehydogenic steroid have also been obtained by incubation of urine at pH 5 with β-glucuronidase after removal of unconjugated steroid and chloroform-soluble conjugated steroid by preliminary chloroform extraction at pH 1. Somewhat similar results have been noted by Kinsella, Doisy & Glick (1950) with an enzyme preparation from *Escherichia coli*, using the Heard-Sobel procedure for determining 'reducing' steroids.

The free formaldehydogenic steroid fraction obtained from the chloroform-insoluble conjugated compounds by the action of β-glucuronidase appears to contain two different types of compound, one of which is slowly destroyed at pH 1 and 37°, the other being relatively stable under these conditions.

This destruction by acid would explain the observation (Pincus & Romanoff, 1950) that continuous extraction with ethylene dichloride during acid hydrolysis at pH 1 yields greater amounts of 'reducing' steroid from urine than are obtainable by extraction after hydrolysis.

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Blood Clotting: the Inhibition by Anions and the Function of Calcium. By J. E. Lovelock and B. M. Burch (introduced by A. Neuberger). (*The Common Cold Research Unit (National Institute for Medical Research), Harvard Hospital, Salisbury*)

The classical theory of blood coagulation includes the idea that the presence of free calcium ions is a necessary condition of coagulation. This idea is based on the circumstantial evidence of the anti-coagulant action of certain ions which are effective in removing calcium ions from solution, namely, oxalate, citrate and fluoride ions. More recently it has been suggested by Quick & Stephanini (1949) and by Overman (1949) that the combined, rather than the ionized, calcium in blood is essential for coagulation.

The results to be reported now suggest that the presence of the element calcium is not essential for the coagulation of blood, and that the anti-coagulant activity of oxalate, citrate and fluoride ions can largely be explained as due to their ionic charge.

The smallest concentration of each of 31 salts needed to prevent the coagulation of human blood was measured. The salts included those with uni-, bi- and ter-valent anions, salts whose anions were effective enzyme inhibitors and salts whose anions were effective in removing calcium ions from solution. There appeared to be no direct connexion between the anticoagulant activity of the salts tested and their ability to remove calcium ions from solution or to inhibit enzyme action. Activity did, however, appear to be closely connected with valency and fell approximately in the order 1:4:20 for uni-, bi- and ter-valent ions respectively. Citrate ions were rather less active than other tervalent ions, oxalate and fluoride ions were rather more active than other bi- and uni-valent ions respectively.

A more direct demonstration that blood would clot in the absence of calcium was made as follows. Calcium-free human and calf plasma was prepared by centrifuging oxalated blood to remove cells and calcium oxalate. This plasma clotted readily on five-fold dilution, or by dialysis with 0·85% NaCl solution. The calcium content of the blood coagulating under these conditions was shown by the use of ^{45}Ca to be only 1% of its normal value. Similar experiments, with similar results, using citrate and fluoride ions, indicated that it is rather the presence of these ions than the absence of calcium which prevents blood containing them from clotting.

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Blood Glutathione and Non-glucose Reducing Substances in Diabetes. By Elizabeth K. B. Illing, C. H. Gray and R. D. Lawrence. (*Department of Chemical Pathology and Diabetic Department, King's College Hospital, Denmark Hill, London, S.E. 5*)

Blood glutathione (both total and reduced) has been determined and compared in 20 normals and 39 diabetics. Eighteen of the diabetics were mild cases and 21 showed severe ketosis. A significant reduction in glutathione was found only in the diabetics with severe ketosis. The glutathione content of blood has been simultaneously compared with the difference between the concentration of 'true' glucose and total reducing substances. Glutathione does not always account for this difference, which suggests that other reducing substances not yet identified must be present in the blood.

The Preparation of Rhodopsin and the Chemical Composition of Rod Outer Segments. By F. D. Collins, R. M. Love and R. A. Morton. (*Department of Biochemistry, University of Liverpool*)

The separation of 'rod' outer segments from the remainder of the retinas has been described by several workers (cf. Collins & Morton, 1950, for a short review). An improved method has been worked out for the preparation of rod outer segments from cattle retinas, which yields rhodopsin solutions of high purity. The method successfully used by Wald & Hubbard (1949) on bullfrog retinas has been found to be less suitable for cattle retinas, owing to their greater friability.

Retinas are shaken with saline and filtered through fine wire gauze. The resulting suspension contains rod outer segments, blood cells, and small tissue fragments. It is poured into a centrifuge tube, and a saturated solution of sucrose is carefully poured down the side so as to form a lower layer in the tube. By carefully stirring at the interface, a gradient of sucrose concentrations is made in the liquid, ranging from saturated sucrose (about 70% w/v) at the bottom to zero at the top.

Rod outer segments are isopycnotic with 30% sucrose, and when the tube is centrifuged they move up or down, until they reach a layer having this concentration. Here they form a dense thin 'band' and are sucked off, diluted with saline, and centrifuged down. They are hardened with a 4% alum solution in water, and extracted with 1% aqueous digitonin. Solutions obtained in this way show the high optical density at 500 mμ. which indicates a good yield of rhodopsin, and a relatively low optical density at 400 mμ. corresponding with freedom from absorbing contaminants.

The isolated rod outer segments have been found to possess the approximate composition (N and P determinations) shown in Table 1.

Table 1. *Composition of isolated rod outer segments*

	% of dry weight
Phospholipin	31·9
Acid-soluble compounds	5·1
Pentose nucleic acids	1·6
Deoxypentose nucleic acids	0·3
Protein	38·5
Remainder (by difference)	22·6
Total	100·0

There is an inverse linear relation between the optical purity of the rhodopsin and the percentage of deoxypentose nucleic acid. The latter is believed to be a contaminant of the rods from retinal nuclear tissue.

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The Regeneration of Rhodopsin. By F. D. Collins. (*Department of Biochemistry, University of Liverpool*)

Zewi (1939) showed that the regeneration of rhodopsin in frog eyes (*in vivo* and *in vitro*) required oxygen. Wald & Brown (1950) and Wald & Hubbard (1950) have reported good regeneration without any apparent need for oxygen. They showed that it was sufficient to add retinene to the bleached rhodopsin solution or to add vitamin A and coenzyme I to the bleached retinas and pigment epithelia. It was implied that the reaction involved the oxidation of vitamin A to retinene (vitamin A aldehyde) by an enzyme which required coenzyme I. The present work was undertaken in order to investigate the effect of oxygen more fully.

It was confirmed that in excised frog eyes, with cornea, lens and vitreous humour removed, regeneration could be obtained. It was also found that cyanide, azide, arsenate, iodoacetate, fluoride, arsenite, malonate and 2:4-dinitrophenol inhibited the regeneration in excised eyes to a greater or lesser extent.

Regeneration could also be observed when the retinas and choroids were removed and ground together in a medium containing various coenzymes and substrates (pH 7·4 phosphate buffer, potassium succinate, coenzyme I, nicotinamide, magnesium chloride, adenosinetriphosphate, and cytochrome *c*) in proportions known to be suitable for the study of oxidative phosphorylation. When the mixture was aerated the amount of regeneration varied from 19 to 54%, but with the addition of a 'solution' of vitamin A in an aqueous detergent the amount of regeneration rose to 70%. Hence vitamin A was subsequently incorporated in the medium. After bleaching the rhodopsin the mixture was aerated for 2 hr. The amount of regeneration was generally in excess of 70%.

Under these conditions a medium containing everything except adenosinetriphosphate produced only 58% as much rhodopsin as the complete medium. Blowing nitrogen through the complete mixture produced 71% as much rhodopsin as with aeration. The activity of the system decreased with time. No inhibition on the addition of 2:4-dinitrophenol could be observed, this result being in apparent conflict with the earlier result.

The need for vitamin A, adenosinetriphosphate and oxygen for maximal regeneration can be regarded as substantiated. Although the need for adenosinetriphosphate does not necessarily imply a novel role, the possibility of producing vitamin A phosphate to act as an intermediate in rhodopsin synthesis merits investigation. The interpretation of the work is complicated by the likelihood of more than one mechanism of regeneration, and the possibility that they operate simultaneously rather than as complete alternatives.

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Quantitative Studies on the Precipitin Reaction using Antigens and Antibodies labelled with Radioactive Isotopes. By G. E. Francis, W. Mulligan and A. Wormall. (*Department of Biochemistry. The Medical College of St Bartholomew's Hospital, London*)

Antigens labelled with ^{35}S (using ^{35}S-containing di-2-chloroethylsulphone for the preparation of 'mustard gas sulphone-protein') and with ^{131}I, and antibodies labelled with ^{131}I have been used in quantitative studies on the precipitin reaction (cf. Boursnell, Dewey, Francis & Wormall, 1947; Banks, Francis, Mulligan & Wormall, 1950*b*). The effect of washing specific precipitates with small volumes of 0·9% NaCl has now been investigated, and it has been found that two washings are sufficient to remove all the non-specific protein and loosely attached antigen and antibody from the precipitates studied; this confirms the observation of Heidelberger & Kendall (1932) made with the aid of total N determinations. We have also found that further washing with 0·9% NaCl does not produce any significant change in the ratio of antibody to antigen in the precipitate.

The antibodies studied can be labelled with up to 1% iodine without serious loss of their precipitating power (Table 1).

The addition of 3:5-diiodotyrosine, in amounts sufficient to effect significant but not complete inhibition of precipitation, causes a marked fall in the antibody/antigen ratio of the precipitates forming in iodinated protein antigen-antibody systems; this is due apparently to the inhibitor reducing the amount of free or effective antibody. It has been found (Banks, Francis, Mulligan & Wormall, 1950*a*) that none of the hapten is combined in these precipitates, and this supports the view that the precipitating antibodies studied are univalent.

As an alternative approach to the study of antibody valence, antigens (ovalbumin and serum-γ-globulins) have been labelled with both ^{35}S and ^{131}I, and the γ-globulin fraction (containing the antibodies) has been prepared from the serum of rabbits injected with 'mustard gas sulphone-iodinated proteins'. These antibodies have been studied to find out whether their combining groups are directed towards either sulphone groups or diiodotyrosine groups, or towards some combination of the two.

Table 1. *Effect of iodination on precipitating antibodies*

Antigen: 'Sulphone-ovalbumin' (ovalbumin treated with ^{35}S-containing di-2-chloroethylsulphone).
Antibody: Iodinated (^{131}I) γ-globulin fraction of rabbit antiserum to 'sulphone-ovalbumin'.
All the N-contents are given as μg. N/ml. antibody solution added.

Iodine content of antibodies (%)	Antigen added (μg. N)	Composition of precipitate: Total N (μg.)	Antigen N (by ^{35}S) (μg.)	Antibody N (by difference) (μg.)	Antibody N (by ^{131}I) (μg.)
0	8·5	107	7·8	99	—
0·33	8·5	109	7·6	101	100
0·65	8·5	102	7·2	95	90
1·17	5·7	88	5·2	83	84
2·51	4·3	36	2·5	33	36

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Studies on Red and White Blood Cells labelled with ^{65}Zn. By R. Tupper, R. W. E. Watts and A. Wormall. (*Departments of Biochemistry and Physiology, The Medical College of St Bartholomew's Hospital, London*)

Keilin & Mann (1939, 1940) found that purified carbonic anhydrase contains zinc, and the role of this element as an active component of the enzyme is fully established (cf. review by Vallee & Altschule, 1949). In our investigations we are using ^{65}Zn to study the distribution of the metal in the blood and other tissues after the injection of small amounts of zinc compounds, and in other experiments we are labelling blood cells (erythrocytes and leucocytes) with this radioactive isotope. The dried blood cells or samples of plasma were digested with acid and ^{65}Zn determinations were made on the acid solution (cf. Banks, Tupper & Wormall, 1950).

For the labelling of the blood cells an acid solution of ^{65}Zn-containing zinc chloride is treated with glycine, and dilute NaOH is added to give pH 7·4; the glycine protects the zinc against precipitation at this pH under the conditions of our experiments. Washed blood cells separated from heparinized blood are then added and the well mixed suspension is kept at 37° for 2 hr. The cells are separated by centrifuging and are washed three times with 0·9 % NaCl; further washings remove no significant amount of ^{65}Zn from these cells.

This labelling with ^{65}Zn appears to cause little damage to the erythrocytes, as judged by microscopic examination and by haemolysis tests with hypotonic NaCl solutions or with haemolysin plus complement.

Separation of the erythrocytes and leucocytes has been effected by the method of Vallee, Hughes & Gibson (1947), which involves flotation on an albumin solution of the right specific gravity. We have found that a solution of dried bovine plasma albumin, fraction V (Armour & Co.), with a specific gravity of 1·064, is satisfactory for rabbit blood, and separation of the labelled cells has revealed that, under the conditions of our experiments, rabbit leucocytes may take up as much as eight times as much zinc as do the erythrocytes.

Some of the ^{65}Zn in the blood cells appears to be fairly firmly bound, but a considerable proportion diffuses out fairly rapidly, possibly as a result of 'exchange', when the cells are exposed to normal plasma. Thus the use of red cells labelled *in vitro* with ^{65}Zn of low specific activity should usually be limited to short-term experiments, but it may be possible, with the aid of carrier-free radioactive zinc and with the biologically labelled cells, to prepare firmly labelled erythrocytes and leucocytes suitable for longer experiments.

When the labelled blood corpuscles were injected into a rabbit the amount of ^{65}Zn in the blood cells of the injected animal decreased rapidly (Table 1). The amount of ^{65}Zn in the blood plasma of this injected rabbit was very small throughout the experiment; thus the zinc liberated from the cells did not appear to accumulate in the plasma.

Table 1. *Distribution of* ^{65}Zn *in the blood of a rabbit after the intravenous injection of* ^{65}Zn*-labelled blood cells*

(Rabbit no. Z16 (2·0 kg.) received 5 ml. of packed labelled cells: the injected material gave a 'count' of 1048/min./ml.)

Time after injection (hr.)	Radioactivity of the blood of the injected rabbit (counts/min.) per g. whole blood	per g. dried blood cells
0·08	38	318
1	30	253
2	24	218
3	19	178
4	16	155
20	3	37
68	1–2	14
121	3	8

A preliminary study has been made of the distribution of ^{65}Zn in the labelled blood cells. The erythrocyte stroma contains very little, and about 90 % of the ^{65}Zn entering the cells is associated with the water-soluble protein fraction, being precipitated by full saturation with $(NH_4)_2SO_4$. Investigations are being made to determine what fraction of the ^{65}Zn is present in the carbonic anhydrase, and the rate of uptake of ^{65}Zn by this enzyme when it is exposed to plasma or salt solutions containing the radioactive isotope.

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Some Constituents of the Unsaponifiable Fraction of Human Sebum. By R. M. B. MacKenna, V. R. Wheatley and A. Wormall. (*Departments of Dermatology and Biochemistry, The Medical College of St Bartholomew's Hospital, London*)

It has been shown (MacKenna, Wheatley & Wormall, 1950) that human sebum contains, on an average, about 29% free fatty acids, 36% combined fatty acids (present in glycerides, waxes and other esters) and 32% of unsaponifiable matter. The unsaponifiable fractions examined contained hydrocarbons, cholesterol and unsaturated alcohols (to the extent, respectively, of about 46, 14 and 8% of the total unsaponifiable matter) and some as yet unidentified compounds. Squalene was identified as one of the major constituents, accounting for about one-third of the total hydrocarbons.

A more complete study has now been made of the unsaponifiable fraction from mixed sebum collected from groups of 30–60 normal male subjects. The sebum was collected by immersing the forearms in acetone, and the acetone-soluble matter was 'purified' by dissolving it in $CHCl_3$, to exclude non-fatty substances present in sweat (this method is a slight modification of that of Ricketts, Squire & Topley, 1948). The unsaponifiable matter was fractionated on Al_2O_3 and the five principal fractions obtained were examined with results given in Table 1. No 'isocholesterol' (lanosterol plus agnosterol) was detected.

No vitamin A, β-carotene, provitamin D_2, K, B_1, or C was detected in sebum. Tests for vitamin E made by various modifications of the colorimetric $FeCl_3$-reducing test (Emmerie & Engel, 1939; Emmerie, 1940) indicated appreciable amounts of tocopherols, but more detailed studies showed that sebum contains several $FeCl_3$-reducing substances. One, an acidic or phenolic compound, is readily destroyed by atmospheric oxidation and it may have a function in sebum as an anti-oxidant. The unsaponifiable fraction of sebum contains two principal $FeCl_3$-reducing substances, or fractions, one being adsorbed on alumina and the other not; part of the latter fraction is destroyed by acetylation indicating the presence of a free hydroxyl group. Some $FeCl_3$-reducing substance is produced on saponification and is probably present in sebum as an ester. It is concluded that sebum normally contains a small quantity of tocopherols but not more than the lipid of most other human tissues.

Table 1. *Fractionation of the unsaponifiable matter of sebum on* Al_2O_3

Fraction	As % of the unsaponifiable matter	Constituents
I	37–46	Squalene. A solid *n*-paraffin (m.p. 57·6°; another preparation 56·2°) (probably pentacosane or a mixture of closely-related hydrocarbons). Remainder (studied by infrared analysis)
II	17–25	Aliphatic alcohols (including one with m.p. 64 or 65·5°; acetyl no. 169). An oxidation product ($C_{30}H_{50}O_4$) of squalene
III	14–19	Cholesterol
IV	4–11	A sterol-like compound plus other unidentified substances
V	6–13	An unidentified amorphous substance (m.p. 79–81°)

Spectrophotometric examination of many of these sebum fractions has been made by Festenstein & Morton (see following abstract).

We are grateful to Dr H. P. Koch and Miss J. M. Fabian of the British Rubber Producers' Research Association for infrared spectrum measurements.

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Spectrophotometric Examination of Sebum Fractions. By G. N. FESTENSTEIN and R. A. MORTON. (*Biochemistry Department, University of Liverpool*)

Free α-tocopherol shows $\lambda_{max.}$ 295 mμ., $E^{1\%}_{1\,cm.} = 77$, and α-tocopheryl acetate shows $\lambda_{max.}$ 285 mμ. $E^{1\%}_{1\,cm.} = 42$. The absorption spectrum of α-tocopherol can be recorded with 0·2 mg. of the vitamin in 4 ml. of solution (1 cm. cell), but accompanying solutes should not exhibit masking absorption. Such conditions are not easily attained if the starting material contains only 0·2 mg./g.

Human sebum showed no well defined selective absorption in the region 230–500 mμ. After chromatography on alumina some weakly adsorbed fractions showed inflexions (250 and 280 mμ.). Although the intensity of absorption at 295 mμ. was greater for the more strongly held fractions, no maximum in the region 285–295 mμ. was observed.

The unsaponifiable fraction from sebum showed faint inflexions in the absorption curve. After chromatography, fractions with definite maxima at 235 and 255 mμ. were obtained. A faint inflexion near 290 mμ. was also observed, but the substance responsible could not be concentrated further and in any case was present in very small amount. A further portion of unsaponifiable matter was subjected to chromatography and yielded fractions showing inflexions between 250 and 270 mμ. The spectrophotometric data imply, since no selective absorption near 295 mμ. was observed, that the maximum amount of α-tocopherol in the unsaponifiable matter could not exceed 0·03 % for the first sample and 0·02 % for the second (expressed as percentage of sebum).

A further concentrate of unsaponifiable material, prepared by Wormall and his colleagues (see the preceding abstract), showed a weak inflexion in the region 260–290 mμ. After chromatography the fractions obtained from it were separately tested for ferric chloride-reducing substances by a modification of the procedure used by Tosic & Moore (1945). The best response was obtained in the final fraction, and if the reduction had been due entirely to α-tocopherol it would have corresponded to 5 % of the observed absorption at 295 mμ. This fraction was therefore chromatographed again, but no maximum at 295 mμ. could be recorded for any resulting fraction. It is therefore unlikely that the main ferric chloride reductant was α-tocopherol. The presence of a little α-tocopherol is not, however, excluded.

At no stage in the work was spectrophotometric evidence obtained for the presence of provitamins D, vitamins A and K or carotene.

A substance with $\lambda_{max.}$ 235 mμ. and another with $\lambda_{max.}$ 278 mμ. (weak) as well, were obtained from a sterol-like product provided by Wormall (see the preceding abstract). Both gave a blue colour ($\lambda_{max.}$ 664 mμ.) with trichloroacetic acid, and also with antimony trichloride in chloroform ($\lambda_{max.}$ 660 mμ.).

In the course of the investigations, squalene, a solid hydrocarbon (m.p. 56°), cholesterol and a substance melting at 79–81° were found to be present, thus confirming the results recorded by McKenna, Wheatley & Wormall (1950).

This work was undertaken at the request of Prof. A. Wormall and we are indebted to him for all the materials used.

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Studies of the Synthesis of Milk Fat by the Perfused, Isolated Bovine Udder. By A. T. COWIE, W. G. DUNCOMBE, S. J. FOLLEY, T. H. FRENCH and R. F. GLASCOCK (*National Institute for Research in Dairying, University of Reading*), L. MASSART and G. PEETERS (*Veterinary School, University of Ghent, Belgium*) and G. POPJÁK (*National Institute for Medical Research, London, N.W.* 7)

Synthesis of milk fat from $CH_3{}^{14}COONa$ by the udder has been shown in the goat (Popják, French & Folley, 1950) and later the cow (Kleiber, Smith & Tolbert, 1950). We have now perfused the isolated bovine udder with $CH_3{}^{14}COONa$ and $NaH^{14}CO_3$ in order to exclude possible incorporation of the labelled carbon into transportable milk precursors in the liver, and to find whether the perfused udder will synthesize milk constituents.

The udder of a lactating cow (East Flemish breed: yield about 12 kg. daily) was perfused for 2 hr. by the method of Peeters & Massart (1947) involving simultaneous perfusion of two separate udder halves. The following additions (at the outset unless otherwise stated) were made to the heparinized perfusion bloods (3 l. for each half at the outset, 2 l. added to each soon after the start): left side: 1800 mg. glucose, 600 mg. sodium acetate containing 1 mc.

Table 1. *Specific activities of glyceride fatty acids and cholesterol from milk and udder tissue after perfusion with* $CH_3{}^{14}COONa$ (1 mc.) *and* $NaH^{14}CO_3$ (1 mc.)

(Specific activities expressed as 1×10^{-3} μc./mg. C.)

Fatty acid fraction		Left side ($CH_3{}^{14}COONa$): specific activity of Milk fat	Left side ($CH_3{}^{14}COONa$): specific activity of Udder fat	Right side ($NaH^{14}CO_3$): specific activity of udder fat
Volatile	water-soluble	3·020	31·450	$3{\cdot}4 \times 10^{-2}$
	water-insoluble	1·590	14·890	$0{\cdot}35 \times 10^{-2}$
Non-volatile	solid	0·334	2·390	$0{\cdot}09 \times 10^{-2}$
	liquid	0·239	1·510	0·000
	Cholesterol	0·028	0·126	

$CH_3{}^{14}COONa$, 80 ml. saline, 25 mg. $NaHCO_3$ in 200 ml. saline added continuously throughout perfusion; right side: 1800 mg. glucose, 600 mg. sodium acetate, 80 ml. D_2O (99·5 %) containing NaCl to 0·9 %, 1 mc. $NaH^{14}CO_3$ (25 mg.) in 200 ml. saline added continuously. The results are summarized in Table 1.

These results considerably reinforce our previous evidence that the mammary gland itself utilizes acetate for milk fat synthesis since this experiment excludes the participation of reactions in the liver. The negligible incorporation of CO_2 from bicarbonate is noteworthy and agrees with the finding of Kleiber *et al.* (1950) in the intact cow. The fact that the specific activity of the udder fat (left side) was about ten times that of the milk fat may be ascribed to dilution of secreted milk with milk pre-existing in the alveoli and ducts. It should also be considered that in the glandular cells the fat as a whole is continuously being turned over. The experiment shows unequivocally for the first time that the perfused udder synthesizes at least one milk constituent.

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Amino-acid Requirements of Amylase Synthesis by Pigeon Pancreas Slices. By L. E. Hokin (Fellow of the American Cancer Society). (*Department of Biochemistry, University of Sheffield*)

In a previous communication (Hokin, 1950) it was shown that slices of pigeon pancreas synthesized α-amylase *in vitro* when incubated in a glucose saline medium, and that addition of a mixture of the twenty common amino-acids increased the rate of synthesis about threefold. In further experiments (Table 1) the ten amino-acids essential for the nutrition of the rat (Rose, 1938) have been found to be as effective in supporting synthesis as the mixture of the twenty amino-acids tested previously*; but when any one of the essential amino-acids was omitted there was a statistically significant reduction in synthesis, except in the case of methionine (Table 2).

* Since this communication was submitted the L-leucine and L-tryptophan used in the above experiments have been found to contain traces of tyrosine. Subsequent experiments with pure leucine and tryptophan indicate that tyrosine is necessary for maximum synthesis.

Boissonnas (1950) has found the following amino-acids in crystalline pancreatic α-amylase in

Table 1. *Synthesis of amylase in the presence of amino-acid mixtures*

(Incubation period 2 hr. at 40°. Bicarbonate saline containing 0·2 % glucose and the indicated amino-acids each in a concentration of 0·02 M, except DL-serine and DL-threonine which were 0·04 M. 5 % CO_2 in O_2. The ten non-essential amino-acids were L-alanine, L-aspartate, L-citrulline, L-cystine, L-glutamate, glycine, L-hydroxyproline, L-proline, DL-serine, and L-tyrosine. The ten essential amino-acids were as listed in Table 2.)

Amino-acids present	Amylase formed per mg. dry wt. (units of Smith & Roe, 1949)
None	20, 19
10 essential amino-acids	62, 64
10 essential plus 10 non-essential amino-acids	62, 56

Table 2. *Effect of omission of individual essential amino-acids on amylase synthesis*

(General conditions as in Table 1. In four of the control experiments the ten essential plus ten non-essential amino-acids were added; in the remaining control experiments the essential amino-acids only were added. Differences in the synthesis of amylase resulting from the omission of amino-acids are expressed in percentages, since the absolute synthesis in different animals ranged from 56 to 186 Smith & Roe units per mg. dry wt.)

Amino-acid omitted	Percentage difference between synthesis in control and deficient mixture of amino-acids: Mean	Range	S.E.M.	No. of experiments
L-Methionine	−4·9	+25 to −29	6·7	8
L-Phenylalanine	−13·2	+7 to −29	4·6	9
L-Isoleucine	−17·9	+15 to −46	6·6	10
L-Histidine	−22·1	−3 to −55	5·4	9
L-Valine	−28·6	−9 to −46	5·4	7
L-Leucine	−31·7	+6 to −58	9·2	6
L-Lysine	−41·8	−15 to −69	7·3	6
DL-Threonine	−55·2	−43 to −74	3·2	6
L-Arginine	−58·7	−39 to −66	4·7	6
L-Tryptophan	−66·3	−50 to −75	4·2	6
All	−60·0	−20 to −76	6·2	8

decreasing order of concentration: aspartic acid, alanine, glycine, leucine and isoleucine, valine and serine, phenylalanine, glutamic acid, threonine, lysine, arginine, proline, histidine, tyrosine and tryptophan. It is noteworthy that methionine is the only essential amino-acid which is absent. The source of the seven non-essential amino acids needed for the synthesis of α-amylase is under investigation.

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The Effect of Thyroxine and Thiouracil on the Metabolism of Carotene. By R. Chanda, M. L. McNaught and E. C. Owen. (*Hannah Dairy Research Institute, Kirkhill, Ayr*)

Thyroxine and thiouracil affect the partition of phosphorus (Chanda & Owen, 1949), the partition of aneurin and the ascorbic acid content of cow's milk (Chanda, McNaught & Owen, 1949), the effect of thiouracil being the reverse of that of thryoxine. The effect of these drugs on carotene metabolism has now been studied. Two rations were used. Ration 2 was a mixture of ground whole oats, bean meal, dried grass meal and 0·1 % Cr_2O_3, with 10 lb. per day of wheat straw as roughage. In ration 1, which contained neither carotene nor Cr_2O_3, the dried grass was replaced by a mixture of blood meal, potato starch and ground wheat straw. The sole source of carotene was the grass meal in ration 2. In periods 1 and 3 (not shown in Table 1), ration 1 was fed to all cows; in periods 2, 4 and 5, ration 2 was fed. In period 4, one pair of cows received thyroxine and a second pair received thiouracil by subcutaneous injection. From the ratio of carotene to chromium in food and in faeces the percentage of undigested carotene was calculated (Chanda, Clapham, McNaught & Owen, 1950). Carotene in food, faeces and milk, and vitamin A in milk, were estimated by the use of chromatography on alumina and a Unicam spectrophotometer. Most of the vitamin of the milk was vitamin A but in Table 1 the carotene in the milk was calculated by assuming 1 i.u. vitamin A to be equivalent to 0·6 μg. β-carotene. Table 1 shows that thyroxine reduced, and thiouracil increased, defaecation of carotene. In the controls carotene appearing in the milk declined as lactation advanced. With the cow Delilah this decline was reversed by thyroxine, while with Griselda it was prevented. With both these cows there was a decrease of the vitamin A activity in the milk on cessation of administration of thyroxine. Changes in the milk produced by the administration and cessation of thiouracil were more marked than those produced by thyroxine, but were in the opposite direction. With thyroxine the ratio of vitamin A to carotene in the milk increased, whereas thiouracil had the reverse effect.

Cama & Goodwin (1949) found that, by mouth, thyroxine decreased, and thiouracil increased, faecal excretion of carotene in the rat. Our results show that these effects are independent of the route of administration of these drugs.

Table 1. *The effect of thyroxine and thiouracil on the metabolism of carotene in cows*

			Carotene metabolism		
				Output as % of intake	
Name of cow	Period no.	Treatment in period 4	Intake (mg./2 days)	Faeces carotene	Milk carotene and vitamin A
Dora	2	None	977	40·7	2·0
	4		978	42·3	1·4
	5		946	45·0	1·4
Tinker	2	None	948	46·0	1·7
	4		947	43·0	1·4
	5		968	44·0	1·3
Delilah	2	Thyroxine	973	45·6	1·7
	4	(10 mg./day)	957	30·2	1·9
	5		957	42·2	1·3
Griselda	2	Thyroxine	963	45·6	1·5
	4	(10 mg./day)	980	26·8	1·5
	5		950	45·5	0·9
Jean	2	Thiouracil	968	42·9	2·2
	4	(20 mg./day)	983	48·9	0·9
	5		929	46·4	1·2
Gwynneth	2	Thiouracil	929	44·5	2·2
	4	(20 mg./day)	973	52·2	0·9
	5		973	43·8	1·2

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The Partition of Vitamin A Activity in Human Milk. By R. Chanda and E. C. Owen (*Hannah Dairy Research Institute, Kirkhill, Ayr*) and Bertine Cramond (*Aberdeen Maternity Hospital*)

Milk from mothers delivered in the Aberdeen Maternity Hospital was analysed for α- and β-carotene and for vitamin A by chromatography on alumina followed by spectrophotometry. The results are recorded in Table 1, which shows that most of the vitamin A activity was present as vitamin A itself, only 7–15 % of it being due to α- and β-carotenes. The total vitamin A activity in all forms was calculated by assuming that 0·6 μg. β-carotene is equivalent to 1 i.u. vitamin A, and that β-carotene is twice as active as α-carotene. There was a wide variation in the total vitamin A activity and

Table 1. *Partition of vitamin A activity in human milk between carotenes and vitamin A, milk yield, fat percentage and output of total vitamin A activity in all forms*

Time *post partum* (days)	Vitamin A (i.u./100 ml.) (*a*)	Vitamin A activity due to α-carotene (i.u./100 ml.) (*b*)	Vitamin A activity due to β-carotene (i.u./100 ml.) (*c*)	Total vitamin A potency (i.u./100 ml.) (*a*+*b*+*c*)	Percentage activity due to carotene	Milk yield (ml.)	Fat (%)	Total output of vitamin A (i.u.)
3	261·2	9·1	29·6	299·9	12·9	130	1·8	390
3	537·1	13·3	43·1	593·5	9·5	363	3·9	2154
3	543·3	10·4	29·7	583·4	6·9	205	3·6	1196
4	238·7	9·1	33·2	281·0	15·1	965	2·9	2712
5	309·8	5·1	22·0	336·9	8·0	651	3·8	2193
6	191·2	3·8	19·0	214·0	10·7	120	1·9	257
7	258·4	3·1	11·5	273·0	5·3	500	3·3	1365
28	218·8	1·8	10·9	231·5	5·5	530	4·2	1227

many of the figures were very large. This wide variation may have been due to failure of some of the mothers to take the capsules of vitamin A and D supplied by the Welfare Centres and the Hospital. Chromatographic separation of vitamin A ester from the alcohol showed that about 25 i.u. (8% of the total) vitamin A was present in the form of the alcohol. This is very much higher than 3 i.u. (3% of the total) recorded for cow's milk by Ganguly, Kon & Thompson (1947).

This work is a portion of an investigation shortly to be published (Chanda, Owen & Cramond, 1950).

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Kinetic Studies of the Conjugation of Phenols with Sulphuric and Glucuronic Acids by the Rabbit. By H. G. Bray and K. White. (*Department of Physiology, Medical School, University of Birmingham*)

We previously found (Bray, Thorpe & White, 1951) that the hydrolysis of benzamide, the oxidation of toluene, benzyl alcohol and benzaldehyde, and the conjugation of benzoic acid with glucuronic acid in the rabbit followed first-order reaction kinetics whereas the rate of conjugation of benzoic acid with glycine was determined by the rate of glycine availability. Our method has been applied to a study of the conjugation of phenols with glucuronic and sulphuric acids. If both these reactions follow first-order reaction kinetics it would be expected that the ratio of the amounts of a phenol excreted as glucuronide and as ethereal sulphate (G/E) would be in the ratio of the first-order velocity constants of the two reactions, (k_g/k_e). If, however, the supply of either conjugating moiety, e.g. sulphate, is restricted, G/E would vary with time. This has been found to be so.

Examination of several phenols showed that their conjugation with glucuronic acid followed the kinetics of a first-order reaction, but values for G/E varied throughout the course of each experiment in which rabbits were maintained on their normal diet, showing that the sulphate conjugation did not follow first-order reaction kinetics under these conditions. Administration of sulphate precursors (e.g. sodium sulphite, cystine), however, could so increase the percentage of the phenol excreted as ethereal sulphate, that a constant value for G/E was obtained throughout the experiment. The same constant values for G/E were obtained by feeding phenol precursors, e.g. anisole. This demonstrates that the sulphate conjugation of phenols takes place according to first-order reaction kinetics only when there is adequate sulphate for conjugation. Thus in experiments carried out under conditions in which there is any deficiency of such sulphate, the percentage conjugation with sulphuric acid, and hence also with glucuronic acid, will be arbitrary, depending on such factors as the nature and quantity of the sulphate precursor in the diet, the dose level and the relation between the times of dosage and of normal feeding. It is clear that values for ethereal sulphate conjugation and hence for G/E are comparable only if the animals are maintained under exceptionally rigidly controlled conditions.

From results of experiments on a series of substituted phenols, values for k_g and k_e have been obtained. There appears to be no relation between the value for k_g and the substituent constant σ of the Hammett (1937) equation

$$\log k - \log k_0 = \rho\sigma,$$

which was derived to express the magnitude of the effect of *m*- or *p*-substituents upon the velocity constants of reactions of aromatic compounds *in vivo*.

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Metabolic Products of the Wood-rotting Fungus *Polyporus benzoinus* (Wahl.) Fr. By E. N. Morgan (introduced by J. H. Birkinshaw). (*London School of Hygiene and Tropical Medicine, London, W.C.* 1)

The investigation of this fungus was undertaken because of the pronounced aromatic odour which it produces in culture. The best growth and yields of metabolic products were obtained by cultivation on an aqueous Vimaltol-Marmite medium.

Ether extraction of the wet, minced mycelium gave a mixture of products. Retreatment of this residue with ether resulted in the separation of an acid (I). The ether filtrate obtained was extracted with aqueous $NaHCO_3$ (2%). On standing, the

bicarbonate layer deposited a crystalline Na salt of a polybasic acid (II). Subsequent evaporation of the remaining ether layer and steam distillation of the residue gave anisaldehyde.

Acid I, m.p. 285–290° (decomp.), $[\alpha]_{5461}^{20°}$ + 8·2 (*c*, 5·1) in pyridine, has the probable empirical formula, $C_{30}H_{46}O_4$. It forms a neutral monomethyl ester, m.p. 192–193°, and a diacetate m.p. 212–214° (decomp.), $[\alpha]_{5461}^{23°}$ + 4·2 (*c*, 6·0) in $CHCl_3$. The diacetate gives formaldehyde on ozonization in $CHCl_3$. Acid I is therefore presumably a dihydroxymonocarboxylic acid with a terminal methylene group. It bears a strong resemblance in properties and colour reactions to the triterpenoid polyporenic acid C, isolated from the natural sporophore of *Polyporus betulinus* Fr. (Cross, Eliot, Heilbron & Jones, 1940; Cross & Jones, 1940). A mixed m.p. determination on the methyl esters of (I) and of acid C (m.p. 190–191°; kindly supplied by Prof. E. R. H. Jones and Dr T. G. Halsall) showed no depression.

Acid II. The Na salt on acidification gives rise to a new crystalline product, $C_{22}H_{38}O_6$, m.p. 78–80°, for which the name *ungulinic acid* is proposed. The acid forms a liquid *dimethyl ester*, $C_{24}H_{42}O_6$, and a *trihydrazide*, $C_{22}H_{44}O_3N_6$, m.p. 190–195° (decomp.). Under different experimental conditions, acidification of the Na salt gives an *anhydride*, $C_{22}H_{36}O_5$, m.p. 53–54·5°. KOH fusion of the Na salt affords stearic, acetic and oxalic acids. Ungulinic acid gives colour reactions which are identical with those described for agaricic acid, $C_{22}H_{40}O_7$. These, and other properties, suggest that ungulinic acid is a lactone of a hydroxytricarboxylic acid.

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Distribution of Injected ^{31}Si in Rats. By P. F. Holt, D. M. Yates and D. H. Tomlin. (*University of Reading*)

The distribution of silica in tissues has been studied by several workers but particularly by King, Stantial & Dolan (1933), who also measured the excretion rate when silicic acid was injected into a rabbit. He found a very rapid excretion rate, but he was unable to recover all the silicic acid which was injected.

We have investigated the distribution of labelled silicon in a number of tissues after intraperitoneal injection of silicate solutions containing the radioactive isotope ^{31}Si into rats.

There is rapid absorption of ^{31}Si from the peritoneal cavity and analyses of the separate tissues have shown no evidence of accumulation in any of the organs investigated other than the kidney. Concentration in the kidney is presumably due to the tubular re-absorption of water and non-absorption of silicate from the glomerular filtrate. This suggestion is supported by autoradiographs of the kidney slices which show general distribution of ^{31}Si throughout the organ but with a higher concentration in the medullary portion where there are tubules only.

The variation of the silica concentration in the kidney both with time and with the amount injected was studied, using the liver tissues as representative of the concentration in other organs of the body.

The time-concentration curve for the kidney after the injection of 8 ml. of a solution containing 1 mg. SiO_2/ml. into rats showed a sharp rise to the 3-hr. value followed by a gradual decline. The liver merely showed a steady drop in concentration down to a value comparable with that found in normal tissues.

Injection of varying amounts of silicate into rats gave an initial sharp rise in kidney silica with increasing dose followed by a rapid fall even to a value below that for the liver.

It would appear that at low concentrations the silicate is concentrated by the kidney and is rapidly excreted. When the silicate is injected in higher concentration the body appears to be unable to deal with it, probably because the silicate is highly polymerized.

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DEMONSTRATIONS

Some Equipment used in the Measurement of Radioactive and Stable Isotopes. By T. E. Banks, V. C. E. Burnop, G. E. Francis, W. Mulligan and A. Wormall. (*Department of Biochemistry, The Medical College of St Bartholomew's Hospital, London*)

Vacuum Manifold used for the Micro-synthesis of Isotopically Labelled Compounds. By V. C. E. Burnop (introduced by A. Wormall). (*Department of Biochemistry, The Medical College of St Bartholomew's Hospital, London*)

The Separation of Leucocytes from Whole Blood by Flotation on Plasma Albumin Solutions (Method of Vallee, Hughes and Gibson). By R. Tupper and R. W. E. Watts. (*Departments of Biochemistry and Physiology, The Medical College of St Bartholomew's Hospital, London*)

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

Volume calibration of apparatus with standard group joints, especially Warburg manometers. By F. DICKENS

Studies on the metabolism of semen. 7. Cytochrome in human spermatozoa. By T. MANN

The specific determination and detection of glucose as a probable constituent radical of certain fructosans by means of notatin. By ANNE PALMER

The fate of certain organic acids and amides in the rabbit. 12. Aminohydroxybenzoic acids. By H. G. BRAY, W. V. THORPE and P. B. WOOD

The metabolism of sulphamezathine, 4:6-dimethylpyrimidine and 2-amino-4:6-dimethylpyrimidine in the rabbit with observations on the hydroxylation on some other sulphonamides. By H. G. BRAY, H. J. LAKE and W. V. THOPRE

Isolated nuclei from cells of the cerebral cortex. Preparation and enzyme content. By D. RICHTER and R. P. HULLIN

Utilization of acetate for milk-fat synthesis in the lactating goat. By G. POPJÁK, T. H. FRENCH and S. J. FOLLEY

The amylase of *Clostridium butyricum*. By W. J. WHELAN and H. NASR

The relationship between the constitution and the effect of chemical compounds on plant growth. 3. Chlorinated benzaldehydes and benzoic acids. By R. L. JONES, T. P. METCALFE and W. A. SEXTON

Selective absorption of stereo-isomers of amino-acids from loops of the small intestine of the rat. By Q. H. GIBSON and G. WISEMAN

Methods for isolating ω-amino-acids: γ-aminobutyric acid from rye grass. By R. L. M. SYNGE

Formation by formaldehyde of a cross-link between lysine and tyrosine residues in wool. By P. ALEXANDER, D. CARTER and K. G. JOHNSON

The virulence-enhancing factor of mucins. 3. Further fractionation of hog gastric mucin; an additional factor in the adjuvant action. By H. SMITH

A statistical evaluation of the lipotropic action of inositol. By C. H. BEST, JESSIE H. RIDOUT, JEAN M. PATTERSON and C. C. LUCAS

The rates of lipotropic action of choline and inositol under special dietary conditions. By C. H. BEST, C. C. LUCAS, JEAN M. PATTERSON and JESSIE H. RIDOUT

The stabilization of D-amino-acid oxidase by flavin-adenine dinucleotide, substrates and competitive inhibitors. By K. BURTON

Biochemistry of fluoroacetate poisoning. Isolation of an active tricarboxylic acid fraction from poisoned kidney homogenates. By P. BUFFA, R. A. PETERS and R. W. WAKELIN

The separation and identification of small amounts of mixed amino sugars. By E. F. ANNISON, A. T. JAMES and W. T. J. MORGAN

The enzymes of carbohydrate metabolism in *Neurospora*. 1. Succinic dehydrogenase. By C. J. SHEPHERD

Removal of acid by trioctylamine from samples of microbiological assay. By D. E. HUGHES and D. H. WILLIAMSON

Absorption and excretion of pheniodol labelled with radioactive iodine. By AUDREY A. FREE, J. E. PAGE and E. A. WOOLLETT

A re-investigation of the question of activators of carbonic anhydrase. By A. M. CLARK and D. D. PERRIN

The reaction of oxidizing agents with wool. 4. The reactivity of tyrosine. By P. ALEXANDER and D. GOUGH

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **293rd Meeting** *of the Biochemical Society was held at the Postgraduate Medical School, Ducane Road, London, W. 12, on Saturday, 27 January, 1951, when the following papers were read:*

COMMUNICATIONS

The Amide and Free Carboxyl Groups of Insulin. By A. C. Chibnall and M. W. Rees. (*School of Biochemistry, University of Cambridge*)

The sample of crystalline Zn insulin (Boots 9633B) used contained (moisture-free) 15·76% N, and the amide N was 9·09% of the total N, corresponding to 12 groups per mol.wt. of 11,700. The weight of glutamic acid and aspartic acid given on acid hydrolysis corresponded to 14 and 6 groups respectively.

The insulin was dissolved in 0·03N 85% (v/v) ethanolic HCl and treated in the cold with an excess of diazo-methane in ether, whereby the free γ-carboxyls of the glutamyl residues, the free β-carboxyls of the aspartyl residues and the free α-carboxyls of the terminal residues were esterified. The resulting product was treated at the boil for 6 hr. with an excess of $LiBH_4$ in tetrahydrofuran (Nystrom, Chaiken & Brown, 1949) to reduce the ester groups to the corresponding hydroxyls. On subsequent acid hydrolysis the above-mentioned glutamyl residues appear as δ-hydroxy-α-amino-*n*-valeric acid, the aspartyl residues as the lactone of γ-hydroxy-α-amino-*n*-butyric acid and the terminal residues as β-amino-alcohols. The latter, having the amino and hydroxyl groups attached to adjacent carbon atoms, will react with periodic acid (Nicolet & Shinn, 1939; Rees, 1946), each amino-alcohol giving rise to 1 equiv. of ammonia, 1 equiv. of formaldehyde and 1 equiv. of an aldehyde characteristic of the amino-alcohol concerned:

$$HIO_4 + RCHNH_2CH_2OH \rightarrow HIO_3 + RCHO + HCHO + NH_3.$$

The hydrolysate of the reduced insulin gave, in excess of the amounts that would be given by the serine and threonine present, 2 equiv. of acetaldehyde, 5 equiv. of formaldehyde and 4 equiv. of ammonia, corresponding to 2 equiv. of β-amino-*n*-propanol, 1 equiv. of aminoethanol and 1 equiv. of an as yet undetermined amino-alcohol. Two of the four constituent chains of the insulin molecule therefore carry terminal alanine residues with the carboxyl groups free, one of the remaining two a similar glycine residue and the other a similar, but as yet unrecognized, residue (cf. Sanger, 1950; Lens, 1949; Fromageot, Jutisz, Meyer & Pénasse, 1950).

The amide groups of the glutaminyl and asparaginyl residues remain unaffected by the esterification and reduction treatments so that on acid hydrolysis they yield glutamic acid and aspartic acid respectively. The hydrolysate of the reduced insulin contained glutamic acid equivalent to 8 groups and aspartic acid equivalent to 4 groups, in agreement with the amide-N, so that the untreated insulin molecule must have contained 8 glutaminyl, 6 glutamyl, 4 asparaginyl and 2 aspartyl residues respectively.

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Paper Chromatographic Separation of Urinary Phenols and Oestrogens. By R. J. Boscott (introduced by H. G. Sammons). (*Department of Anatomy, University of Birmingham*)

Background fluorescence from urinary constituents may interfere with the fluorometric analysis of urinary oestrogens (cf. Engel, Slaunwhite, Carter & Nathanson, 1950). The application of paper chomatographic methods to this problem has therefore been investigated.

The following solvent systems have been employed for the separation of phenolic acids and

oestrogens, using Whatman no. 54 and 542 filter papers:

Stationary phase	Mobile phase
1. Propylene glycol	Benzene
2. Propylene glycol-ethylene glycol mixtures	Benzene, benzene-ethyl acetate mixtures
3. Ethylene glycol	Ethylene dichloride
4. Glycerol	Acetone, ethylmethyl ketone, methylene dichloride, ethylene dichloride, chloroform, amyl alcohol
5. Capryl alcohol	10% $KHCO_3$, 10% K_2CO_3

Moore & Stein (1948) reported the use of a starch-0·1 N-HCl adsorption chromatogram for the separation of amino-acids. Samec (1932) has reviewed the adsorptive properties of cellulose.

Paper chromatograms employing aqueous alkali as the mobile phase, and no organic solvent, have now been found to give good separations of phenolic acids, oestriol and fluorescent components of urinary oestriol fractions. Aqueous pyridine and triethanolamine buffered with K_2HPO_4 also gave good resolution of urinary phenolic acid mixtures. Whatman no. 542 paper and the ascending method were used.

The possibility of two-dimensional adsorption chromatography using aqueous alkalis is under investigation.

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The Cortisone-Iodine Complex. By G. T. Bassil (*Department of Obstetrics and Gynaecology, University of Manchester*) and R. J. Boscott (*Department of Anatomy, University of Birmingham*) (introduced by H. G. Sammons)

Zaffaroni, Burton & Keutman (1950) reported that cortisone interacted with an aqueous solution of iodine to give a blue-coloured complex. No other steroids investigated by these authors gave the reaction.

Chromatographic analysis of products obtained from (1) periodate oxidation of cortisone, and (2) alkaline hydrolysis of cortisone acetate, revealed the presence of compounds other than cortisone (A) giving a positive reaction with the iodine reagent.

Androst - 4 - en - 17α - ol - 3 : 11 - dione - 17 - carboxylic acid (B), but not adrenosterone (C) formed a blue complex with iodine. Pure reference compounds of other degradation products of cortisone reported by Mason (1938) were not available for investigation.

Cholic acid (D) was unique among the many other reference steroids studied by us, in also giving a positive iodine reaction (Barger & Field, 1912; Barger & Starling, 1915).

The blue iodine complexes from (B) and (D), but not (A), were found to be bleached by saturated $NaHCO_3$ solution containing I_2. The iodine complex from (A) was stable only in the presence of an excess of I_2. KI solution and organic solvents dissociated the blue steroid-iodine complexes.

The rate of formation of the iodine-steroid complex from pure crystalline (A), (B) or (D) is accelerated when the crystalline structure is broken down. (A) reacts more rapidly with I_2 in the presence of H_2O_2 or $(NH_4)_2S_2O_8$. The iodine-steroid complexes when examined microscopically revealed blue needle crystals and long narrow blue fibres, as well as blue amorphous material. The crystals and fibres exhibited pleochroism indicative of the production of a solid solution or clathrate-type compound. The breakdown of the usual crystalline form of the parent steroid appears to be essential for the production of the iodine complexes. The blue complex formed from (A) after H_2O_2 treatment is largely due to the presence of a reactive acidic derivative resembling (B).

In spot tests on paper chromatograms the production of a blue colour by cortisone is readily inhibited by a number of compounds, e.g. fructose, adrenal lipids.

Alternative tests for (A), (B) and (D) on paper chromatograms include the following. (1) Reaction with N-NaOH solutions of triphenyltetrazolium compounds: (A) produces a pink spot due to formazan formation; (B) and (D) do not react. (2) Sulphuric acid 90% gives a vivid green fluorescence with (B). (3) Dinitrophenylhydrazine (1%) in methanol containing 1% conc. HCl gives orange-pink spots with (A) and (B). No reaction is given by (D). (4) Using benzene-formamide on Whatman paper no. 542, Zaffaroni *et al.* (1950) in partition chromatography of (A), (B) and (D) by the ascending method, the following R_F values were obtained: (A), 0·09–0·10; (B) and (D), 0–0·05.

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A Mucoid from Sheep Submandibular Glands Inhibiting Haemagglutination by Influenza Virus.

By J. F. McCrea. (*The Lister Institute of Preventive Medicine, London, S.W.* 1)

Several mucoids of human or animal origin have been shown to inhibit the specific agglutination of erythrocytes by influenza virus, and hence to act as competitive substrates with the 'receptor substance' on the red cell surface (Burnet, McCrea & Anderson, 1947; Burnet, 1948; Svedmyr, 1949). Purified human serum mucoid (McCrea, 1948*a*) and a phenol-insoluble mucopolysaccharide from ovarian cyst fluid (McCrea, 1948*b*) were active inhibitors of heated influenza B virus, but the lability of the former mucoid and the rarity of the latter prevented their preparation on a scale suitable for thorough chemical examination.

An extremely active electrophoretically homogeneous mucoid has now been isolated from submandibular glands of sheep. The excised glands were stored for 3 months in absolute alcohol; they were then minced, extracted in water in a Waring blender, heated to 80° for 10 min., and stood overnight at 0°. After centrifugation at 8000 rev. per min. in an angle centrifuge the supernatant fluid was freeze-dried. Fractionation of a 10% solution yielded a heavy precipitate with 2 vol. of ethanol, and a light flocculent precipitate with between 3 and 4 vol. of ethanol. The first precipitate was dissolved in borate buffer at pH 6·8 and deproteinized by a modified Sevag method. A snow-white fluffy material resulted on freeze-drying: yield 1·32 g. from 458 g. of glands.

The purified mucoid dissolves rapidly to give a water-clear solution. The ninhydrin and biuret tests are strongly positive and the Millon and Molisch tests weakly positive. Analysis: N, 10·13; P (organic), 0·33; reducing sugar as glucose, 13·6; hexosamine, 12·4 (Elson & Morgan, 1933), 16·7% (Dische & Borenfreund, 1950). The Morgan & Elson (1934) test for *N*-acetylhexosamine is positive, the ultraviolet absorption curve of the colour obtained being identical with that of authentic *N*-acetylhexosamine. The hexosamine component is galactosamine only. A trace of fucose is present. Electrophoresis at pH 4 and 8 shows a very sharp single peak on the ascending side and a broader asymmetrical peak on the descending side; at both pH the mucoid migrates approximately twice as fast as serum albumen.

As little as 0·005 μg. of the purified mucoid completely inhibits 5 agglutinating units of heated influenza B (LEE) virus in the standard test with fowl erythrocytes. The activity against several 'indicator' A strains (Stone, 1949) is considerably lower.

The inhibitory power of the mucoid is rapidly lost on contact with active virus or the receptor-destroying enzymes of *Vibrio cholerae* (Stone, 1947) and *Clostridium welchii* (McCrea, 1947). Dilute trypsin and potassium metaperiodate also rapidly inactivate the mucoid.

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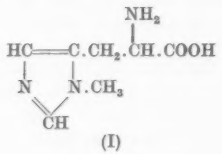
The Occurrence of Free Methylhistidine in Urine. By J. M. SEARLE and R. G. WESTALL. (*Medical Unit, University College Hospital Medical School, London, W.C.* 1)

In routine studies of normal and pathological urines using paper chromatography, an unknown ninhydrin-reacting substance has frequently been found which moved to a position close to that occupied by proline. This substance was believed to be an unknown amino-acid and was referred to as 'green-spot' in the 'map of spots' recorded by Dent (1948). Methylhistidine, i.e. β-(3-methyl-4-glyoxalinyl)-alanine, (I) was plotted separately in a neighbouring position, but we are now certain that they are, in fact, the same substance:



(I)

That their identity was not recognized sooner may have been due to the variable colour of the ninhydrin-reaction spot which gives a brown colour at temperatures below 100° and only goes green when the paper is heated to about 110°. Recently Dr S. P. Datta and Dr H. Harris (private communication) found that cat urine contains relatively large amounts of 'green-spot', and we have used this source for its isolation.

The cat urine was fractionated on a column containing the cation-exchange resin ZeoKarb 215 using the procedure described by Partridge (1949). The successive fractions collected during displacement of the amino-acids with aqueous ammonia were examined by paper chromatography, and those which contained 'green-spot' were bulked and refractionated on a column containing the anion-exchange resin Dowex II (Davies, Hughes & Partridge, 1950) using 0·1 N-HCl for development. The selected fractions containing 'green-spot', which were free from any other ninhydrin-reacting substance, were bulked and evaporated to about 5 ml.; addition of 3 vol. of ethanol yielded a crop of colourless crystals. This material was recrystallized twice. (Found: C, 49·0; H, 6·5; N, 24·7. Calc. for $C_7H_{11}O_2N_3$: C, 49·7; H, 6·6; N, 24·8%.) It was chromatographically identical with methylhistidine derived from a sample of anserine. 'Green-spot' m.p. 246–247°; methylhistidine m.p. 245–247°; mixed m.p. 245–247° (all uncorr.), and in each case with decomposition. The substance gave no colour with diazotized sulphanilic acid.

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Vitamin B_{12}. Additional Factors. By E. LESTER SMITH, K. H. FANTES, S. BALL, D. M. IRELAND and J. G. WALLER (*Research and Development Division, Glaxo Laboratories Ltd., Greenford, Middlesex*) and W. B. EMERY, W. K. ANSLOW and A. D. WALKER (*Fermentation Division, Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Bucks*)

The following red crystalline substances, all microbiologically and clinically active, have been isolated from *Streptomyces griseus* fermentation liquors: vitamin B_{12}, vitamin B_{12a} and vitamin B_{12b} (now known to be identical), vitamin B_{12c} (Anslow, Ball, Emery, Fantes, Lester Smith & Walker, 1950), vitamin B_{12d} (Anslow *et al.* 1950).

These are believed to be present as such in the fermentation liquor, since their isolation can be achieved by physical methods alone. Other factors have, however, been prepared by mild chemical treatment of these (see below). The yields of the new factors, vitamins B_{12c} and B_{12d}, were enhanced at the expense of vitamin B_{12b} by treating the eluate from a charcoal adsorbate of the fermentation liquor with nitrous acid. This step was introduced into the purification procedure to deaminate amino-acids and peptides and thereby facilitate their removal.

The ready interconvertibility of vitamins B_{12c} and B_{12d} was eventually explained by the presence of a nitrite group in the molecule of vitamin B_{12c}, presumably co-ordinated with the cobalt in the same way as the cyanide group similarly situated in vitamin B_{12} itself (Veer, Edelhausen, Wijmenga & Lens, 1950; Brink, Kuehl & Folkers, 1950; Kaczka, Wolf, Kuehl & Folkers, 1950). However, nitrous acid treatment appears to alter the structure in some other way also, for removal of the nitrite group (by distillation with dilute acid or treatment with sulphamic acid) gives vitamin B_{12d} and not vitamin B_{12b}. These factors are both basic and have identical absorption spectra, but differ in their behaviour

both on partition chromatograms and in the plate method for microbiological assay.

In electrical conductivity and polarographic behaviour vitamin B_{12c} resembles vitamin B_{12} itself. Its specific optical rotation is, however, $+50°$ compared with $-110°$ for vitamin B_{12} and -20 and $-15°$ for vitamins B_{12a} and B_{12d} respectively (all at 643·8 mμ.).

Treatment of vitamin B_{12} with sulphite gives rise to a substance with principal absorption maxima at 364 and 515 mμ.: it appears to incorporate sulphite in the molecule, though this is readily lost on crystallization or on distillation of aqueous solutions under reduced pressure. The product is vitamin B_{12a}, identified by its absorption spectrum and chromatographic behaviour (see also Fricke, Lanius, De Rose, Lapidus & Frost, 1950).

Vitamin B_{12c} behaves similarly, the nitrite group being displaced by sulphite. Conversely, the sulphite adduct is converted into vitamin B_{12c} by excess nitrite.

In a somewhat similar manner, alkaline cyanide solutions convert any of the vitamin B_{12} factors into a purple substance having principal absorption maxima at 368 and 580 mμ. This cyanide adduct presumably incorporates two cyanide groups, since one is already present in vitamin B_{12}. The second is lost very readily; for example, the colour returns to red in a few minutes merely on acidifying, and the spectrum is then that of vitamin B_{12}.

Other substances will form adducts with the vitamins B_{12}, e.g. thiocyanate, thioglycollate and iodine. This adduction is sometimes evidenced by changes in absorption spectrum and usually by a marked increase in extractability into solvents such as benzyl alcohol or phenol-benzene mixtures.

The adducts appear to be weakly acidic in character, for they are more readily extracted from acid than from alkaline solutions; the basic factors vitamins B_{12a} and B_{12d} show the opposite behaviour.

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Vitamin B_{12}. Excretion Studies. By D. C. Chesterman, W. F. J. Cuthbertson and H. F. Pegler.
(*Research and Development Division, Glaxo Laboratories Ltd., Greenford, Middlesex*)

The urinary excretion of vitamin B_{12} after oral or intramuscular administration has been studied in ten normal male subjects and one pernicious anaemia (P.A.) patient. All estimations were carried out by a modification of the method of Bessell, Harrison & Lees (1950).

In agreement with Chow, Lang, Davis, Conley & Ellicott (1950) we observed that the main elimination occurred within 8 hr. of injection. We found that the amount excreted in this time varied with the dose given, e.g. after injection of 1000 μg., from 330 to 470 μg. were excreted, while after 50 μg. injections 3·2–4·1 μg. were eliminated. In a series of 20 experiments employing doses ranging from 5 to 1000 μg. the relationship between dose given and amount excreted could be accurately defined by the expression

$$E = D - 1{\cdot}2D^{0{\cdot}89},$$

where E is the amount of B_{12} excreted after 8 hr. and D is the amount of B_{12} injected intramuscularly.

After oral administration excretion is small, e.g. after oral doses of 9 and 1–3 mg. about 1 and 0·03–0·2 μg., respectively, were excreted over the next 24 hr. Ungley (1950) showed that 3 mg. doses of B_{12} given orally to P.A. patients led to responses similar to those expected after the injection of about 100 μg. The urinary excretion encountered in our work was lower than might be anticipated from the clinical observations. Further work is in progress to investigate these findings.

Saturation tests. In two normal subjects the daily administration of 20 or 50 μg. B_{12} led to a rise in urinary elimination; 2 days after stopping the injections the excretion dropped to that found after the first. In one P.A. patient (not in relapse) excretion of B_{12} after injection of 20 μg. was less than that found in normal subjects; continued daily injections did not increase B_{12} excretion to the extent noted in a normal person. The conclusion may be drawn that under our conditions the B_{12} reserves may be increased, but that these increased reserves are not retained for more than 2 days.

We wish to thank Dr J. N. M. Chalmers for access to the pernicious anaemia patient, and Mr B. Basil for mathematical assistance.

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On the Cyanide Inactivation of Succinic Dehydrogenase and the Relation of Succinic Dehydrogenase to Cytochrome *b*.

By C. L. Tsou. (*The Molteno Institute, University of Cambridge*)

The dehydrogenases were considered to be insensitive to cyanide until Dixon & Keilin (1936) discovered that xanthine dehydrogenase is slowly but irreversibly inactivated by incubation with cyanide. In the present investigation it is shown that succinic dehydrogenase is affected in the same way.

On incubation of the heart-muscle preparation (Keilin & Hartree, 1947) with cyanide, succinic dehydrogenase, estimated by the Thunberg method using methylene blue (MB) or dichlorophenolindophenol as hydrogen acceptor, was slowly but irreversibly inactivated. The rate of inactivation depended upon the temperature of incubation and the cyanide concentration; the inactivation invariably went to completion if the enzyme was allowed to remain in contact with cyanide for a sufficiently long time. By plotting the time for 50% inactivation against the reciprocal of cyanide concentration a straight line was obtained showing that the reaction involved one molecule of cyanide per molecule of enzyme. From the slope of the straight line the reaction velocity constant was calculated to be 0·097 l./mol./min. at 22°. The rate of inactivation was independent of the pH of incubation, thus indicating that the enzyme reacted with hydrocyanic acid and not with cyanide ion.

The identity of succinic dehydrogenase with cytochrome *b* has been suggested by Bach, Dixon & Zerfas (1946) and by Ball, Anfinson & Cooper (1947) and has recently been supported by Pappenheimer & Hendee (1949). Results obtained in the present study, however, lead to the view that (1) succinic dehydrogenase is the immediate hydrogen or electron acceptor of succinate and only the oxidized form of the enzyme reacts with cyanide, (2) succinic dehydrogenase is not identical with cytochrome *b*. The evidence in support of this conclusion can briefly be summarized as follows: (1) the enzyme is protected from the action of cyanide by its substrate, succinate (Table 1), and also by sodium dithionite. The minimum concentration of succinate to give complete protection was 2 mM. and that required to give a measurable protection was 0·02 mM. Malonate (50 mM.), fumarate (50 mM.) and pyrophosphate (10 mM.), all known to have a high affinity for the enzyme, were ineffective, as were cystine (2 mM.) and cysteine (4 mM.). (2) When the enzyme is incubated with cyanide and succinate at a concentration sufficient only to give a partial protection, not only is the reaction rate reduced but the inactivation remains incomplete. (3) No straight line relationship exists between the intensity of the α-band of ferrocytochrome *b* and the succinic dehydrogenase activity. (4) After almost complete inactivation of the dehydrogenase, cytochrome *b* can still be oxidized and reduced by various means. (5) All cases of cyanide inhibition of a haem-protein are reversible; no irreversible inactivation of a haem-protein by cyanide is known.

Table 1. *Inactivation of succinic dehydrogenase by cyanide*

(Experiments were carried out in Thunberg tubes, one of them (no. 4) with two hollow stoppers. The tubes contained 0·1 ml. dehydrogenase, 0·04 M-succinate; 0·0003 M-MB; 0·02 M-KCN; and 0·1 M-phosphate buffer, pH 7·3. When KCN in solid form was added (5 mg.) in one of the hollow stoppers, an equivalent amount of sulphuric acid (0·7 ml., 0·1 N) was added to the other. Volumes were made up to 4 ml. with water. After being allowed to stand at room temperature for 4 hr., the tubes were evacuated and warmed to 38°. The contents of the main tubes and the hollow stoppers were mixed and the reduction time measured.)

Tube no.	Main tube	Hollow stoppers		Reduction time	
		A	*B*	min.	sec.
1	Enzyme + buffer	MB + succinate	—	2	25
2	Enzyme + buffer + HCN + succinate	MB	—	2	30
3	Enzyme + buffer + HCN	MB + succinate	—	28	—
4	Enzyme + buffer	MB + succinate + H_2SO_4	KCN	2	20

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The Effect of Processing Methods on the Availability of Lysine in Groundnut Meals. By K. J. Carpenter and G. M. Ellinger. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Groundnut meal, an important source of protein, has to be heated during the process of oil extraction. Meals have been found to vary in their nutritive value with the severity of the processing (Cama & Morton, 1949; Mitchell, Hamilton & Beadles, 1949). Lord & Wakelam (1949) have suggested that these differences may be explained by the destruction of organic sulphur compounds.

It has been shown with other natural feeding stuffs and with protein-carbohydrate mixtures that lysine may be destroyed or made unavailable by heating (Patton, 1950) and by storage in a humid atmosphere (Lea & Hannan, 1950). We have set out to determine whether this could account for the difference found by Cama & Morton.

significantly in their lysine content, the figures agreeing with those obtained by microbiological assay (De Man, 1949).

The availability of lysine was tested in growth trials with chicks by the method of Grau (1946), who compared test materials as the sole protein source in a synthetic diet. In each trial every ration was fed to duplicate groups of eight chicks for a 10-day period.

The groundnut meals were fed to give 20 % protein in the diets and were all supplemented with 0·3 % DL-methionine. Lysine was then the limiting factor in the diets, and the addition of 0·3 % L-lysine gave a growth rate equal to that of a stock diet. The mean growth rates in the three trials are shown in Table 1.

Table 1. *The effect of processing methods on the availability of lysine in groundnut meals*

Sample no.	Treatment of groundnuts	Lysine (g./16 g. N)	No. of growth trial	Mean growth of chicks in 10 days: Diet without added lysine (g.)	Mean growth of chicks in 10 days: Diet with added lysine (g.)	Difference
1	Ether extracted	3·4	—	—	—	—
4	Commercially screw expelled, solvent extracted and dried at a low temperature under reduced pressure: solvent removed with minimal steam	3·3	1	85	102	+17
			2	73	94	+21
			3	69	84	+15
5	Meal no. 4 heated dry at 150–160° for 1 hr.	3·2	2	61	83	+22
6	Meal no. 4 twice soaked in water and dried at 100°	3·2	3	66	84	+18
3	Commercial extraction as for meal no. 4 but dried at a higher temperature: solvent removed with heavy steaming	3·1	1	78	98	+20
			3	80	102	+22

The method of preparation of the samples is given in Table 1. Enzymic assays were carried out on the samples after acid hydrolysis (5 g. refluxed in 50 ml. 6N-HCl for 24 hr.) using the decarboxylase method of Gale (1948). Under these conditions tests showed that the addition of starch to casein or casein and lysine mixtures did not affect the recovery of lysine. Lysine added to groundnut samples before hydrolysis was completely recovered. The meals did not differ

No significant differences were found within single trials between the meals tested. The absolute growth without added lysine, from comparison with other data (Grau, 1946), suggests that the major portion of the lysine remains available in all the meals.

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On the Nature of Naturally Occurring Porphyrin Precursors. By C. H. Gray. (*Department of Chemical Pathology, King's College Hospital, London, S.E.* 5)

Waldenström & Vahlquist (1939) believed that the porphyrin precursor porphobilinogen, which is excreted in acute porphyria, is a mixture of two dipyrrylmethenes. The only evidence supporting this view was that the molecular weight as measured by a diffusion method was about 350.

The ultraviolet absorption spectrum of porphobilinogen solutions, repeatedly purified by chromatography on alumina (Prunty, 1945), shows a maximum at 282–284 mμ. Dipyrryl-methenes show two absorption maxima, a stronger one in the region of 450 mμ. and a weaker one in the region of 300–320 mμ. It therefore appears that porphobilinogen is a mixture, not of dipyrryl-methenes, but either of dipyrryl-methanes or of monopyrrolic compounds, both of which possess a maximum in the region of 260–280 mμ. It is possible that porphobilinogen is related to the monopyrrolic precursors postulated by Neuberger, Muir & Gray (1950). *In vivo* such monopyrrolic precursors would be enzymically condensed to give a naturally occurring mixture of types I and III isomers, while *in vitro* the condensation would not be enzymically controlled and there would result a complex mixture of porphyrins of the type observed by McSwiney, Nicholas & Prunty (1950) and by Gibson & Harrison (1950). The varying degrees of carboxylation encountered in the complex porphyrin mixture obtained *in vitro* would be expected from loss of CO_2 from one or both side chains either prior to, or during, condensation.

Raine (1950) has described an ether-soluble precursor of coproporphyrin in urine. Studies of the urine from a case of acute porphyria suggest that this ether-soluble precursor might be a metal complex of coproporphyrin. Ether extracts of the urine in question were red in colour and showed a typical metal-complex spectrum with maxima at 575 and 540 mμ. (intensity I > II). Only small quantities of coproporphyrin could be extracted from the ether layer with 0·15N-HCl, and only on standing could further quantities be obtained with this acid. However, when extraction was performed with stronger acid the metal-complex spectrum immediately changed to that of porphyrin in acid solution. The porphyrin then passed rapidly and completely into the acid phase, from which coproporphyrin III was finally isolated in the form of its tetramethyl ester. The ether-soluble precursor reported by Raine may therefore be such a metal complex, usually present in insufficient concentration to give a recognizable absorption spectrum in the hand spectroscope, but in sufficient quantity for the porphyrin from it to be determined by fluorescence.

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Some Results with the Estimation of Plasma Protein-bound Iodine. By R. FRASER and P. SCARLETT (introduced by E. J. KING). (*Postgraduate Medical School, London, W.* 12)

Plasma protein-bound iodine estimations have been done by a slightly modified Chaney method (Chaney, 1940); protein precipitation by $ZnSO_4$ and NaOH, digestion in $KMnO_4$ and H_2SO_4, distillation by adding oxalic acid and trapping in NaOH, colorimetry by ceric sulphate-arsenious acid.

The accuracy of the different steps in the method, especially in colorimetry and distillation, have been assessed. Duplicate results on cases grouped by a clinical assessment of their thyroid function were presented to indicate the usefulness of the method clinically.

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Studies of Porphyrin Metabolism in Disorders of the Blood-forming Organs. By J. C. WHITE and S. VENTURA. (*Postgraduate Medical School, London, W.* 12)

The levels of free erythrocyte protoporphyrin (EP), serum iron (SI), iron-binding capacity and serum copper have been determined in a series of thirty-five patients with various blood disorders. In twenty-six of these, the urinary coproporphyrin, and the faecal coproporphyrin and protoporphyrin excretion, have been studied during the same period. A small group of normal individuals is also under examination.

EP was determined by the method of Grinstein & Wintrobe (1948). Urinary coproporphyrin was separated by the method of Dobriner & Rhoads (1938), and the faecal porphyrins soluble in ether-acetic acid were fractionated after the procedure of Dobriner (1937). The optical density of hydrochloric acid solutions was measured in the Beckman spectrophotometer, using wavelengths at the absorption maximum of each porphyrin in the Soret band region (401 mμ. for coproporphyrin in 0·15N-HCl; 409–411 mμ. for protoporphyrin in 2–3N-HCl). In addition to the position of the absorption maximum in this spectral region, the use of paper chromatography, as described by Nicholas & Rimington (1949), was very useful in establishing the homogeneity of the fractions.

As standards, a sample of pure protoporphyrin-IX methyl ester (m.p. 226–228° corr.,) was prepared from haemoglobin (Grinstein, 1947), and coproporphyrin was prepared from porphyric faeces.

A clear-cut relationship between elevated EP (> 100 μg./100 ml. of packed erythrocytes) and low SI values (< 60 μg./100 ml.) obtained in most cases of iron-deficiency anaemia; with treatment, gradual rise of SI followed haematological improvement, but decline of EP was variable and slight. In haemolytic anaemias of various types, higher SI values were found (> 120 μg./100 ml.), but the EP was variable and not clearly related to the reticulocyte levels. In some severe disorders of the reticulum and leucocyte-forming tissues (Hodgkin's disease, leukaemias, myeloma, etc.), there was a shift towards low SI and high EP values.

The urinary and faecal coproporphyrin excretion was very variable, and seldom exceeded the published normal range (Dobriner & Rhoads, 1940; Watson & Larson, 1947). On the other hand, although the faecal protoporphyrin varied considerably, rates of excretion higher than 50 μg./day were often found in blood disorders, usually in conjuction with high EP. The diets of these patients were not controlled, however, so that the endogenous contribution is subject to argument.

In eight samples of faeces, chloroform-soluble pigments with the characteristic chlorin-type spectrum, showing strong absorption at 668 mμ. (Rabinowitch, 1944), were found, and were presumably derived from the dietary chlorophylls.

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Metabolic Response *in vitro* to Electrical Stimulation of Sections of Mammalian Brain. By H. McIlwain. (*Biochemical Laboratories, Institute of Psychiatry, Maudsley Hospital, London, S.E.* 5.)

When suitable potential changes are brought about in electrodes at or near the intact brain *in situ*, physiological responses occur which suggest the functional activity of the brain to have increased. The electrical activity of the brain itself also increases. Associated with these changes are rapid alterations in chemical constituents of the brain, which include the breakdown of labile phosphoric esters. The rates of respiration and glycolysis of the brain, as determined by arterial and venous difference, also increase. To understand the relationship between the electrical changes and these chemical changes which appear to support them, it is desirable to try to examine them in as simple a system as possible. It was found recently (McIlwain & Cheshire, 1950) that thin sections of mammalian brain cortex could be caused to resynthesize labile phosphoric esters, and thus this prerequisite for response to stimulation can be provided *in vitro*. Under somewhat similar circumstances, Libet & Gerard (1939) had observed electrical activity in fragments of frog brain *in vitro*.

Suitable electrodes and vessels have been devised by means of which electrical contact can be made to thin sections of mammalian brain cortex, while these are immersed in nutrient salines and shaken in an atmosphere of oxygen. The tissue-holding electrodes do not at all lower the respiratory rate. The application for some hours of a.c. at up to 3 V., or condenser discharges of up to 50 V. for a few msec. several times per sec., caused in the apparatus no changes which might be mistaken for metabolic responses. When brain-cortex slices were in proximity to electrodes at which such electrical changes were occurring, the respiration of the slices increased. Changes to up to double those of the initial respiration were observed. The increased respiration remained within the range of metabolic activity of the brain in enriched and Ca-free media recently reported by Krebs (1950).

The increased respiration could be maintained for some hours, and in a given experimental arrangement increased with increasing voltage of the current applied, when this was above a threshold value. The increase in respiration was largest when the proportion of the tissue which was lying fairly closely between the electrodes was greatest, and in a given experimental arrangement it changed with the character of the electrical stimulus. The change in many cases paralleled the sensitivity of the intact brain to different types of electrical stimuli.

Other metabolic changes have been observed in brain tissue under these conditions (McIlwain, Anguiano & Gore, unpublished) and in the presence of added substances. Other tissues have been studied and several behave differently (Kratzing & McIlwain, unpublished).

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The Interaction of Serum Globulins with Calcium. By N. H. Martin and D. J. Perkins. (*Department of Chemical Pathology, St George's Hospital Medical School, London, S.W.* 1)

Martin & Perkins (1950*a*, *b*) described experiments designed to investigate the interactions between calcium and various purified albumins. The same techniques have now been extended to certain globulin fractions from pooled normal human sera. The present work deals with two fractions: (*a*) a study of globulin purified to contain not less than 90–95 % γ-globulin, (*b*) a fraction referred to here as the 'β-globulin fraction'; this is a mixture of proteins having mobilities of the order of α-, β- and γ-globulins, the predominant component migrating in the range of the β-globulins. It is derived from the precipitate resulting when 9 % by vol. of ether is added to a solution of a mixture of globulins at a pH 5·0 in a 0·01 M buffer. It is the supernatant from this buffer from which the purified γ-globulin is obtained (Kekwick & MacKay, 1949). The fractionation at pH 5·0 is very sensitive to slight changes in conditions difficult to avoid in large-scale production.

Table 1 shows the mean values of four batches of γ-globulin. Two batches of crude dried material gave bindings of 0·7 mg. Ca/g. N or less. Previous experience with albumins suggests that this may have been the result of modification during processing. There is nothing to suggest that calcium binding is in any way related to the immunological properties of γ-globulin.

Table 2 shows values for five 'β-globulin fractions' tested. These show considerable variation in their ability to bind calcium though individual fractions

were reasonably constant in their behaviour whether examined fresh, after freeze-drying and storing, or after extracting with ether at −20° (Table 3).

Table 1. *Calcium binding capacity of human serum γ-globulins of* 90–95 % *purity by electrophoresis*

Sample no.	pH studied	Range of protein conc. studied (g./l.)	Ca (mg.) / Protein N (g.) (mean of readings)
1	6·7–6·8	2·6–8	3·9
2	6·5–6·6	1·6–3·3	4·2
3	6·5–6·6	4·3	4·2
4	6·7–6·9	4·6–7	3·8
		Mean of 16 analyses	4·0

Table 2. *Variations in calcium binding capacity of five 'β-globulin' fractions*

Sample no.	pH	Protein N mg. in sample	Ca (mg.) / Protein N (g.)
1	6·8	9·6	10·6
2	6·7	14·0	3·4
3	7·5	12·2	3·0
4	6·8	16·0	1·9
5	7·5	6·9	0·7

Chanutin, Ludewig & Maskett (1942), from their studies on the binding of calcium to protein, concluded that between pH 6·5 and 8·0 fluctuations in pH were not associated with significant changes in binding. All the studies here are within those pH. While fluctuations in pH may play a part in the variations shown in different 'β-globulin fractions', it is considered that it is variations in composition that are of major importance. The type of electrophoretic analysis obtained is not capable of satisfactory resolution but the tracings of three of the samples studied suggest a relation between the amount of the slower moving components of the β-fraction and the extent of binding. It would appear strange that the binding of the γ-fraction is low, when some of the evidence suggests that it is the slower component of the 'β-fraction' that gives a high binding value. It is very possible, however, that such material would tend to remain in solution when the γ-globulin is finally precipitated from the supernatant at pH 6·7.

Table 3. *Calcium binding capacity of human serum β-globulin sample no.* 1

	pH	Protein N (mg.) in sample	Ca (mg.) / Protein N (g.)
(*a*) Fresh	6·7	6·2	11·0
	6·7	6·2	11·6
(*b*) After freeze-drying and storing	6·7	6·4	10·8
	6·8	12·8	10·7
(*c*) After ether extraction at −20°	6·4	4·1	13·5
	6·5	8·1	13·5

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The Estimation of Lactic Acid in Silage. By A. J. G. Barnett. (*Division of Agricultural Biochemistry, Department of Biological Chemistry, University of Aberdeen*)

Classical methods for the determination of lactic acid in silage suffer from a variety of drawbacks. That due to Foreman (1920) provides merely an estimate of total non-volatile fatty acids of which lactic acid is but the chief component. Again, the method of Hirsch-Kauffman (1924), elaborated by Karström & Virtanen (1937), involves the removal, by a complicated and lengthy procedure, of proteins and soluble carbohydrates and ends with an iodometric titration. Other iodometric methods which have been employed involve the use of solutions which require to be renewed daily.

The method of Barker & Summerson (1941) for blood lactic acid has been used for the determination of lactic acid in milk and milk products by Davidson (1949), but proteins and soluble carbohydrates have to be removed in these cases. This method, which is extremely sensitive and depends on the fact that lactic acid is oxidized by sulphuric acid to acetaldehyde which gives a deep red colour with *p*-hydroxydiphenyl in the presence of copper ions, has been applied to the determination of the lactic acid content of silage. The procedure is straightforward because with the amount of fresh material used in the extraction (5 g. in 100 ml. water) only in extreme cases could there be as much as 0·25 % of protein in the extract. This small amount can be removed with the copper hydroxide-calcium hydroxide suspension simultaneously with the soluble carbohydrates. The recovery is high, ranging from 96 to 102 %. As will be seen from Table 1, the percentage of lactic acid in silage diminishes rapidly with rise of pH, the critical pH for the continued survival of the acid in silage being about 4·2.

Table 1. *The lactic and content of silage*

pH	Lactic acid content of fresh material (%)	Dry-matter content of silage (%)	Lactic acid content calculated on basis of dry-matter content (%)
3·84	1·442	18·20	7·92
3·90	1·625	25·00	6·50
3·94	1·320	25·63	5·15
4·05	0·856	16·19	5·29
4·26	0·902	15·82	5·70
4·27	0·767	15·75	4·87
4·30	0·489	17·49	2·80
4·49	0·457	15·23	3·00
4·50	0·486	17·20	2·82
4·65	0·202	16·20	1·25
4·96	0·319	14·38	2·22
4·99	0·303	12·98	2·33
5·54	0·213	18·25	1·17
5·60	0·075	15·00	0·50

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Hyaluronic Acid in Exudate from Oedematous Sexual Skin in Rhesus Monkeys. By K. G. Rienits (introduced by D. F. Cole). (*Department of Anatomy, University of Birmingham*)

The observations of Ogston, Philpot & Zuckerman (1939), Chain & Duthie (1940) and Duran-Reynals, Bunting & van Wagenen (1950) suggest that hyaluronic acid, or hyaluronic acid with an admixture of chondroitin sulphate, is present in the extracellular exudate from oedematous sexual skin of rhesus monkeys. This material has now been shown to contain hyaluronic acid with no detectable amounts of chondroitin sulphuric acid.

Incubation of the exudate with streptococcal hyaluronidase (which does not hydrolyse chondroitin sulphuric acid) causes complete loss of turbidity or clot formation on acidification to pH 3–4. The partially purified polysaccharide upon hydrolysis with 2N-HCl contains no detectable sulphate, and gives positive tests for hexuronic acids and hexosamines. Using paper partition chromatography the hexuronic acid was shown to have the same R_F values as glucuronic acid. The identification of the hexosamine was only accomplished by oxidative degradation with ninhydrin to a pentose as described by Gardell (1950). The pentose was identified by paper chromatography as arabinose without any trace of lyxose, showing that the parent hexosamine was glucosamine without any chondrosamine. No sugars other than glucuronic acid and glucosamine could be shown to be present. The presence of hyaluronic acid in extracellular oedema fluid is unusual except in inflammatory oedema and must arise from the action of oestrogens upon sexual skin tissue.

The degree of polymerization of hyaluronic acid can be roughly estimated by the nature of the precipitate obtained on acidifying to pH 3–4 in the presence of protein. By this test it has been found that the degree of polymerization is greater in the small, hard swellings than in the large, very oedematous swellings. It is suggested that during the sexual skin cycle the hyaluronic acid undergoes a cycle of depolymerization and polymerization.

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DEMONSTRATIONS

A Counter-Current Apparatus. By G. H. LATHE and C. R. J. RUTHVEN. (*The Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London, W.* 6)

A fifty-tube counter-current apparatus has been constructed from glass and plastic. Individual glass tubes are connected alternately by large-bore and small-bore plastic tubing of the Polythene type. The whole apparatus is set up as a spiral about a central horizontal support which rotates on its long axis. By suitable rotation of this support two liquid phases in each tube may be equilibrated, allowed to separate, and the heavier moved on by one tube leaving the lighter behind. This apparatus is designed to take a maximum of 20 ml. of solvent in each phase. Smaller amounts, or phases of different volume, may be used. Approximately 3 hr. are required to make a counter-current distribution of fifty tubes. The distribution of a solute is found to be in close agreement with the theoretical, calculated from the partition coefficient.

This apparatus is particularly useful in lipid fractionation, since emulsions which frequently form are immediately evident, and, if necessary, the contents of a tube may be drawn off through a side opening for centrifuging. The capacity of the tubes is larger than that of Craig's apparatus (Craig, 1944). Morever, the size of the apparatus could readily be increased both with regard to number of tubes and their capacity. The apparatus can be built very economically.

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Normal Biochemical Values for Whole Blood and Serum. By I. D. P. WOOTTON, E. J. KING, I. MACLEAN SMITH and RUTH M. HASLAM. (*Postgraduate Medical School, London, W.* 12)

In most cases normal values are published as a single range. This is demonstrably inadequate as a description of a frequency distribution because different ranges may be established depending upon what percentage of results are to fall within the range. For this reason, we have determined the normal values of various biochemical constituents of the blood of healthy individuals. These were determined under routine conditions by the standard methods used here (King, 1951), and must be considered strictly applicable only to this laboratory.

Blood samples were obtained from volunteers from both sexes between the ages of 18 and 50. All the subjects had been at work for at least the 2 months previous and were healthy, i.e. were not known to be suffering from systemic disease. No food or drink was taken during the 4 hr. preceding the sampling. From the results (50–100 values were available for each constituent) frequency distributions were plotted and curves fitted by a graphical method.

As shown in Table 1, a number of distributions were fitted with a 'normal' curve. Almost all the remainder, however, were positively skewed and were fitted with curves of the 'log-normal' type. Such curves have frequently been found appropriate to biological data (Gaddum, 1945*a*, *b*). The serum globulin results showed negative skewness and limits were fitted by inspection.

The asymmetric nature of most of the distributions makes impossible their specification by mean and standard deviation. Instead, four levels have been listed for each constituent: the outer or 1% limits enclose 98% of the distribution, 1% falling below the lower level and 1% rising above the upper. The inner of 10% limits similarly enclose 80% of the distribution.

The application of Table 1 is a matter of opinion. It is suggested that individual results falling outside 10% limits should be viewed with suspicion, while results falling outside the 1% limit may be considered abnormal.

Table 1. *Blood constituents of normal persons, 10 % and 1 % limits*

(For further explanation, see text.)
(All results are in mg./100 ml.; except phosphatase, in units; amylase in units; and protein, in g./100 ml. Numbers in parentheses are m-equiv./l.)

Estimation	Lower		Upper		Distribution
	1 %	10 %	10 %	1 %	
Whole blood:					
Chloride (as NaCl)	581 (99)	593 (101)	620 (106)	632 (108)	Normal
Cholesterol	115	140	215	265	Log-normal
Creatinine	0·1	0·1	1·2	2·6	Log-normal
Glucose	55	68	96	109	Normal
Non-protein N	25	29	43	51	Log-normal
Urea	12	16	35	47	Log-normal
Uric acid	0·6	1·6	3·9	4·9	Normal
Serum:					
Amylase	71	91	163	209	Log-normal
Bilirubin	0·1	0·1	0·5	0·8	Log-normal
Calcium	9·1 (4·5)	9·6 (4·8)	10·9 (5·4)	11·4 (5·7)	Normal
Alkaline phosphatase	3·3	4·5	9·5	12·9	Log-normal
Acid phosphatase, total	0·8	1·2	3·1	4·6	Log-normal
Acid phosphatase, formaldehyde stable	0	0	2·1	4·1	Log-normal, with many zero values
Total protein	6·3	6·7	7·7	8·2	Normal
Albumin	4·0	4·4	5·3	5·7	Normal
Globulin	1·5	1·9	2·8	3·0	Negatively skewed
Potassium	13·5 (3·5)	15·1 (3·9)	19·6 (5·0)	21·7 (5·6)	Log-normal
Sodium	306 (133)	316 (137)	340 (148)	350 (152)	Normal

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Trichloroacetic Acid Precipitation Method for Lipid Phosphate. By E. J. King and W. Weedon. (*Postgraduate Medical School, London, W.* 12)

The plasma proteins contain negligible quantities of phosphorus. When the plasma proteins are precipitated with trichloroacetic acid the phospholipins are quantitatively precipitated with them, and estimation of the total phosphate contained in the trichloroacetic acid precipitate from plasma, therefore, yields the same result for lipid phosphate as the determination of the phosphate in an ethanol-ether extract (cf. Zilversmit & Davis, 1950).

Plasma (0·2 ml.) is diluted with water (1 ml.) in a centrifuge tube, and trichloroacetic acid (5 ml. of 5 % w/v) is then slowly added to the tube while the contents are well mixed with a swirling motion. After 5 min. the precipitate is centrifuged, the supernatant fluid decanted, and the tube well drained on filter paper. Perchloric acid (60 %) (0·5 ml.) is now added and the tube heated over a very small flame, or, preferably, on an adjustable electric heater (see following abstract). The addition of a small piece of carborundum is very effective in preventing bumping. The mixture turns black and then brown and finally becomes colourless when the digestion is complete. The tube is removed from the heater and cooled. 5 ml. of water are added followed by 0·4 ml. of 5 % (w/v) ammonium molybdate and 0·2 ml. of reducing agent (aminonaphthol-sulphonic acid) (see King, 1951). The blue colour so produced is compared with that of a suitable standard containing, e.g., 0·02 mg. P.

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An Electric Heater for Micro-Kjeldahl, Organic Phosphate and Other Acid Digestions. By E. J. KING and W. WEEDON. (*Postgraduate Medical School, London, W.* 12)

The heater consists of two banks of electric coil elements running through porcelain units which are so constructed as to form an easy mount for test tubes, small Kjeldahl flasks and centrifuge tubes. These elements are obtained from a small electric stove which is commercially available.

The electric heater is best operated with the front heaters at low and the back row at high until all the water has been driven off, and the digestion mixture begins to fume as the acid becomes concentrated. Thereafter, the front row is turned to high, and the back row is turned off until the digestion is complete. During the initial stages of the heating, when water is being driven off, the back row of heaters maintains the barrel of the test tube or flask at a sufficient temperature to prevent condensation of drops of water which would cause severe spattering if they were to fall back into the hot concentrated acid.

Interference Strip Spectrum Light Filter for Photoelectric Colorimeter. By E. J. KING and W. WEEDON. (*Postgraduate Medical School, London, W.* 12)

A graded strip filter covering the visual spectrum (mounted in glass 15 × 4 cm.) was obtained from Barr and Stroud Ltd., Glasgow, W. 3. A set of determined spectral points (kindly measured by Mr R. B. Donaldson of the National Physical Laboratory) is marked on a scale attached to the filter to indicate the mean wavelength of light for each segment being used. The filter is mounted in a slide rack between the light source and the glass cuvette and photoelectric cell. The light is passed through one of a set of metal slides, with different slit widths, to control the wave band of spectral light. It is necessary to use a wider slit at the violet end of the spectrum than at the red end, because of the greater optical density of the filter there.

By means of this strip filter a simple photoelectric colorimeter is easily converted into an instrument which is virtually a spectrophotometer. The isolation of a desired portion of the spectrum is more quickly achieved, and there is a higher transmission for a narrower band of spectrum than is possible by the use of absorption light filters of glass or gelatin.

Cloudy Perspex Standards for the Turbidimetric Estimation of Protein and Chloride. By E. J. KING, T. A. WEBSTER and J. HASLAM. (*Postgraduate Medical School, London, W.* 12, *and Plastics Division, Imperial Chemical Industries Ltd., Welwyn Garden City, Herts.*)

Light Filters of Coloured Perspex. By E. J. KING and S. VENTURA. (*Postgraduate Medical School, London, W.* 12)

Determination of Copper and Zinc in Blood Serum. By S. VENTURA and E. J. KING. (*Postgraduate Medical School, London, W.* 12)

Heylmeyer & Plotner (1937) first introduced 6N-HCl extraction as a simple method of freeing iron from the serum proteins, thus making the Fe ion available for colorimetric determination. Many other chemical substances, such as HNO_3, trichloroacetic acid (TCA), sodium citrate, and HCl of different concentrations have been used, but 6N-HCl extraction remains the easier and most certain method for the purpose. Confirming the *in vitro* researches of Surgenor, Koechlin & Strong (1949) with the 'β_1-metal-combining globulin', Holmberg & Laurell (1949) showed that serum contains a globulin, caeruloplasmin, which is able to bind Cu in the same way as siderophyllin binds Fe. This evidence suggested to us that 6N-HCl might free the Cu from its linkage with the serum proteins. This is much simpler and quicker than the procedure of Cartwright, Jones & Wintrobe (1945), which involves three extractions of the serum with 20% TCA at 90–95°. Treatment with 6N-HCl for 10 min., followed by protein precipitation with 20% TCA, and by extraction of the coloured compound of Cu with sodium diethyldithiocarbamate (1%) in ether-amyl alcohol (1 : 1), yielded similar results to the longer and more complicated TCA-triple extraction method, with which they agreed with a coefficient

of variation (C.V.) of 2·33 % (sixteen bloods from four subjects). Recovery of added Cu averaged 97 %.

The same 6N-HCl extraction has been used as a simplification of the complex procedure described by Hoch & Vallee (1949) for Zn in plasma, which uses dithizone in CCl_4 for the colorimetric determination. The nature of the compound containing Zn in serum is unknown; certainly it is not bound to the enzyme molecule of the carbonic anhydrase as in erythrocytes. But we suggest, as Surgenor *et al.* (1949) showed *in vitro* with the 'β_1-metal-combining globulin', that Zn is bound to proteins in a simple transport fraction, like Fe and Cu. Our results, using this simplified procedure, agreed with those obtained by the method of Hoch & Vallee, with a C.V. of 0·7 %. Recovery of added Zn was 98·5 %.

The 6N-HCl is the most satisfactory concentration for liberating the Cu and Zn; 4N-HCl does not free all the metal bound to protein; 8N-HCl also fails to free it completely, perhaps because of the too rapid precipitation of the proteins.

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The Effect of Compounds Allied to Resorcinol upon the Uptake of Radioactive Iodine (^{131}I) by the Rat Thyroid. By D. G. Arnott and I. Doniach. (*Postgraduate Medical School, London, W.* 12)

Following the discovery of the antithyroid properties of resorcinol in humans (Bull & Fraser, 1950) and its effect of lowering the uptake of ^{131}I by the rat's thyroid (Doniach & Fraser, 1950), a series of phenolic compounds was tested in an attempt to relate chemical structure to antithyroid action. The rats (average wt. 160 g.) were tested in groups of four, each rat receiving 0·1 mmol. of the compound assayed. Ten minutes after subcutaneous injection of the drug, the rats were given 10 μc. of ^{131}I intraperitoneally and killed 2 hr. later. The thyroids were then removed, digested with sodium hydroxide in aqueous ethanol, and their radioactivity measured in a liquid counter. The iodine uptake was compared with that of the thyroids of rats treated with ^{131}I alone.

In these comparisons, polyhydric phenols and their derivatives fell into three groups as follow: (1) Substances which produced gross reduction of thyroid activity, e.g. resorcinol, 1 : 3-dihydroxynaphthalene, phloroglucinol. (2) Substances which in some experiments produced slight reduction, at other times none, e.g. pyrogallol, 1 : 5-dihydroxynaphthalene. (3) Substances which produced no reduction at all, e.g. hydroquinone, catechol. Monohydric phenols and their derivatives invariably produced either slight or no reduction.

These results suggest that this type of antithyroid effect is associated with phenols of a resorcinol-like structure.

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Calcium Method for 0·5 ml. of Serum. By E. J. King, Dorothy Bain and Valerie Pash. (*Postgraduate Medical School, London, W.* 12)

The procedure is best carried out with a Conway microburette containing 0·015N-potassium permanganate. Serum (0·5 ml.) is treated in a 5 ml. conical centrifuge tube with 0·5 ml. of water and 0·5 ml. of saturated ammonium oxalate solution. The contents of the tube are thoroughly mixed, and centrifuged after 30 min. The supernatant fluid is carefully poured off and the tube allowed to drain on filter paper. The precipitate is washed with 2 ml. of dilute ammonia solution so that the mat of calcium oxalate at the bottom of the tube is thoroughly stirred. A little of the dilute ammonia is washed down the sides of the tube, which is then centrifuged and drained as before. The washing is repeated. The precipitate is dissolved in 0·5 ml. N-H_2SO_4 in a beaker of hot (80°) water. It is then titrated hot with the 0·015 N-$KMnO_4$, while the solution is stirred by bubbling CO_2 or air from a fine tube. Since 1 ml. of 0·015N-$KMnO_4 \equiv$ 0·3 mg. Ca; therefore mg. calcium/100 ml. of serum = ml. 0·015 N-$KMnO_4 \times 60$.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **294th Meeting** *of the Biochemical Society was held at the London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C.* 1, *on Saturday,* 17 *February* 1951, *and took the form of a Symposium on* '*The Biochemistry of Fertilization and the Gametes*'.

COMMUNICATIONS

Fertilization *in vitro* of the Mammalian Egg. By AUDREY U. SMITH. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Fertilization comprises the penetration of the egg by the spermatozoon, and the subsequent formation and syngamy of the male and female pronuclei. The morphological and biochemical changes involved are of great academic and practical interest, and several different methods have been used for studying them. Many attempts have been made to effect the process *in vitro* with mammalian gametes, but, in spite of occasional claims of success, no consistently effective technique has so far been worked out.

To achieve fertilization *in vitro* with mammalian material rigid technique for collecting unfertilized eggs is essential and, since they are readily activated to segment parthenogenetically, strict criteria of fertilization must be adopted. The only certain proof of *in vitro* fertilization would be to observe and photograph the spermatozoon penetrating the vitellus, and the subsequent formation and fusion of male and female pronuclei.

The classical studies of Schenck, of Onanoff, and of Pincus and his co-workers on rabbit and guinea-pig eggs, and those of Meakin and Rock on human ovarian eggs, fail to satisfy these criteria. Moricard extirpated, and injected semen into, the fallopian tubes of virgin rabbits soon after induced ovulation. After incubating the tubes and their contents *in vitro* he fixed and sectioned them, and demonstrated spermatozoa within eggs, but not formation of pronuclei, syngamy, or segmentation. Austin, using mated rats, recovered eggs soon after sperm penetration. He studied and photographed by phase-contrast microscopy the development and fusion of male and female pronuclei. The complete process of *in vitro* fertilization remains unaccomplished.

Mammalian Semen: Composition, Metabolism and Survival. By T. MANN. (*Molteno Institute, University of Cambridge*)

Semen as ejaculated is composed of two parts, the spermatozoa and the seminal plasma. Spermatozoa are generated in the testes and stored in the epididymides where they undergo a 'ripening process' accompanied by characteristic metabolic changes. Mature spermatozoa are endowed with two highly specialized functions, fertility and motility. The head of the spermatozoon contains a high concentration of deoxyribonucleic acid; it can be separated mechanically from the middle piece and tail. The spermatozoa carry a wealth of enzymes and co-enzymes, including the complete cytochrome and glycolytic systems. On the other hand, they possess a relatively small intracellular reserve of nutrients, and depend to a large extent on nutrients derived from two extracellular sources, the seminal plasma and the secretions of the female genital tract. The seminal plasma is a composite mixture of secretory fluids contributed by the male accessory glands. It is distinguished from other body fluids by the presence of a high concentration of fructose, citric acid, choline, phosphorylcholine, and a number of highly active phosphatases and proteolytic enzymes.

Anaerobically, spermatozoa metabolize fructose to lactic acid. Aerobically, the fructolysis is followed by secondary oxidative processes. Spermatozoa are also able to oxidize various organic acids, lipids and amino-acids (with hydrogen peroxide as product). They are capable of surviving for a long time outside the male body. Under storage conditions *in vitro*, the survival and metabolism of sperm can be prolonged by means of suitable artificial diluents.

Hormonal and Nutritional Factors in the Metabolism of the Male Accessory Organs of Reproduction. By CECILIA LUTWAK-MANN. (*Biochemical Laboratory, University of Cambridge*)

The male accessory organs of reproduction in mammals consist of several glands which produce certain specific secretions; these secretions make up the seminal plasma, the natural medium for the ejaculated sperm cells. In contrast to the extensive and detailed histological studies, there was until fairly recently a scarcity of data concerning the chemical composition and the metabolic properties of these organs, but of late a considerable amount of information has been accumulated, particularly as regards the prostate, seminal vesicle and coagulating gland. A turning point in the biochemical studies was reached when it was found that the metabolism of the accessory glands, as expressed in their secretory function, is strictly dependent upon the male sex hormone.

It is possible to demonstrate by means of various experimental techniques the dependence of the secretory ability of the accessory organs upon the testicular hormone, the classical example being gonadectomy, followed by involutionary changes, and then full restitution of the secretory function by means of testosterone administration. Since the endocrine activity of the testes is controlled by the anterior pituitary gland, hypophysectomy leads in time to changes identical to those due to castration; recovery in the accessory glands of hypophysectomized animals can be achieved by means of gonadotrophin.

So strictly is the metabolism of the accessory glands subject to hormonal regulation, that it is possible to utilize the assay of certain constituents of the seminal plasma as a *sui generis* 'hormone indicator test'.

The production, release and stability in the body of hormones, are influenced to a considerable extent by the nutrition of the animal. Therefore, apart from the direct hormonal regulation, the metabolism of the accessory glands is also profoundly modified by the nutritional state of the male organism. This explains the far-reaching, though not irreversible, changes which take place in the accessory glands in conditions of dietary insufficiency.

Hyaluronidase and Fertilization. By G. I. M. SWYER. (*University College Hospital Medical School, London, W.C.* 1)

Freshly ovulated mammalian ova are surrounded by masses of follicle cells—the cumulus—which, it has generally been supposed, must first be dispersed before spermatozoa can reach and fertilize the eggs. McClean & Rowlands (1942) showed that hyaluronidases from several sources could produce such an effect; they suggested that this was the purpose of seminal hyaluronidase and further that the reason why apparently very large numbers of spermatozoa were essential for fertility was that it was necessary for a sufficient concentration of the enzyme to be established in the vicinity of the cumulus before its dissolution and subsequent fertilization of the contained ova could take place. The experiments of Rowlands (1944) suggested that added hyaluronidase could increase the fertilizing capacity of dilute rabbit-semen suspensions.

Several subsequent workers showed that in most Mammalia the concentration of seminal hyaluronidase is related to sperm density, that it is the spermatozoa which carry the enzyme and that its release into the surrounding medium appears to depend upon fairly simple physico-chemical relationships. In birds and reptiles, in which Orders the eggs are not surrounded by cumuli, seminal hyaluronidase is apparently absent. These facts were all consonant with the theory expressed above.

However, attempts to demonstrate a therapeutic value, in human infertility due to oligospermia, for hyaluronidase when used after the fashion of Rowlands, were unsuccessful, and Chang (1947) showed that it was not the hyaluronidase, but the seminal plasma containing it, which was the effective agent in Rowlands's experiments; it was, in fact the 'dilution phenomenon' (Emmens & Swyer, 1948) wherein spermatozoa in very dilute suspension (i.e. of the order of 0·5 million/ml.) rapidly lose their motility, with which Rowlands had been dealing. The final blow to the theory came with the demonstration by Austin (1948), using the phase-contrast microscope, of male and female pronuclei and sperm tails within newly fertilized rat eggs still surrounded by follicle cells. It must therefore be concluded that the hyaluronidase carried by a spermatozoon enables it to burrow its individual passage through the intercellular substance of the cumulus and that subsequent disintegration of the latter is a relatively unimportant side effect. It is unlikely that the role of hyaluronidase in fertilization can be invoked in explanation of the necessity for the insemination of large numbers of spermatozoa to ensure normal fertility.

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Sperm-Egg Interacting Substances and Metabolic Changes Associated with Fertilization. By Lord Rothschild. (*Department of Zoology, University of Cambridge*)

Sperm-egg interacting substances, or gamones, are concerned with the activation, inactivation and agglutination of spermatozoa, with the precipitation or dissolution of the jelly surrounding eggs, and with the liquefaction of the egg cortex. One of these substances, derived from eggs, is claimed to have a chemotactic effect on homologous spermatozoa, but in the animal kingdom the evidence in support of this view is inadequate. In ferns, mosses and seaweed, chemotaxis of spermatozoa towards eggs or archegonia occurs.

The chemical changes which take place in sea urchin eggs at fertilization include acid production, a fall in R.Q., reduction in glycogen content, ammonia accumulation, release of calcium, increase in uptake of inorganic phosphorus, and fluctuations in the activity of certain enzymes. Increases in oxygen uptake at fertilization are unlikely to be significant under natural conditions. Egg cytolysis is also associated with acid production; phloridzin inhibits the formation of this cytolytic acid, which is claimed to be phosphoric acid.

Contrary to earlier views the cytochrome system functions both in fertilized and unfertilized eggs. Carbon monoxide photo-reversibly inhibits the respiration of unfertilized and fertilized eggs.

The pathways of carbohydrate metabolism in sea urchin eggs are still uncertain. In some species, carbohydrate appears to be broken down through a glycogenolytic cycle; in others, an oxidative mechanism proceeding through phosphogluconic acid has been postulated.

Biochemistry of Sexual Reproduction in Plants. By Lilian E. Hawker. (*Department of Botany, University of Bristol*)

The lower plants do not present so many difficulties in a study of the biochemistry of sexual reproduction as do the flowering plants, consequently more exact data relating to the former are available.

(1) *Fungi.* Sexual reproduction in many fungi is stimulated by aneurin and/or biotin which also promote mycelial growth. Additional specific spore-promoting substances are sometimes required, e.g. for maturation of zygospores of *Phycomyces blakesleeanus* (factors Z_1 and Z_2, the former replaceable by guanine or hypoxanthine).

Heterothallism in fungi is probably correlated with the synthesis by the two opposite strains of complementary spore-promoting substances. The mode of action and chemical nature of these has been studied in *Achlya* spp.

(2) *Algae.* Hormones controlling sex, flagellation and conjugation of gametes have been isolated and identified with certain carotenoids. Carotene is also present in motile male gametes of certain brown algae and aquatic fungi and in the reproductive parts of some moulds.

(3) *Ferns, mosses and liverworts.* Spermatozoids are positively chemotactic to a number of substances, including malic acid, carbohydrates, proteins and some inorganic salts, some of which may be present in the archegonia.

(4) *Flowering plants.* Experiments on vernalization and photoperiodism indicate the existence of a flower-promoting hormone or 'florigen'. It is uncertain whether this is identical with, related to or additional to the known plant growth regulators of the auxin group.

Pollen tubes are positively chemotropic to substances contained in the ovules or ovary wall.

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

The coupled oxidation of pyruvate with glutathione and cysteine. By D. CAVALLINI

The effect of amino-acids on the growth of *Lactobacillus casei* A.T.C. 7469 in pteroyl glutamic acid-free media. By E. G. HARRY

The action of some water-soluble poly-α-amino-acids on blood clotting. By A. DE VRIES, A. SCHWAGER and E. KATCHALSKI

The isolation of αε-diaminopimelic acid from *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*. By ELIZABETH WORK

End-group assay in some proteins of the keratin-myosin group. By K. BAILEY

The size, shape and aggregation of tropomyosin particles. By T.-C. TSAO, K. BAILEY and G. S. ADAIR

Studies on the analysis of vitamins D:

1. The reactions of vitamins D and related substances with iodine trichloride. By J. GREEN
2. The analytical purification of vitamin D by differential solubility, precipitation reactions, and chromatography. By J. GREEN
3. Experiments on the separation of vitamin D from vitamin A and other substances by condensation techniques and selective irradiation. By J. GREEN

Ester formation by yeasts:

1. Ethyl acetate formation by *Hansenula* species. By R. DAVIES, E. A. FALKINER, J. F. WILKINSON and J. L. PEEL
2. Formation of ethyl acetate by washed suspensions of *Hansenula anomala*. By J. L. PEEL

Comparative studies of 'bile salts'. 2. Pythocholic acid. By G. A. D. HASLEWOOD and VERYAN M. WOOTTON

Cholesterol metabolism. 3. Cholesterol metabolism in the guinea pig and rabbit. By R. P. COOK and R. O. THOMSON

Vitamin A in the kidney of the rat. By EVA EDEN and T. MOORE

*allo*Pregnane-3α : 20α-diol in the urine of pregnant women. By T. I. KYLE and G. F. MARRIAN

The biochemistry of locusts:

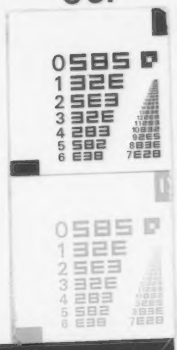
6. The occurrence of a flavin in the eggs of a pterin in the eyes of the African migratory locust (*Locusta migratoria migratorioides* R. & F.) and the desert locust (*Schistocerca gregaria* Forsk.). By T. W. GOODWIN and S. SRISUKH
7. A note on the effect of breeding temperature on the carotenoid content of locusts (the African migratory locust, *Locusta migratoria migratorioides* R. & F. and the desert locust, *Schistocerca gregaria* Forsk.). By T. W. GOODWIN

Purification of horse-radish peroxidase and comparison of its properties with those of catalase and methaemoglobin. By D. KEILIN and E. F. HARTREE

Addendum—Determination of sedimentation and diffusion constants of horse-radish peroxidase. By R. CECIL and A. G. OGSTON

Studies on the enzymic synthesis of glutamine. By W. H. ELLIOTT

On the mechanism of the inhibition of glycolysis by glyceraldehyde. By DOROTHY M. NEEDHAM, L. SIMINOVITCH and SARAH M. RAPKINE

Antithyroid substances. 2. Some mercaptoglyoxalines, mercaptothiazoles and thiohydantoins. By C. E. SEARLE, A. LAWSON and H. V. MORLEY

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